



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/31, C07K 14/315, 7/08, A61K 38/10, 39/09, G01N 33/566</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 99/45121</b> <b>(43) International Publication Date:</b> 10 September 1999 (10.09.99)
<b>(21) International Application Number:</b> PCT/US99/04326 <b>(22) International Filing Date:</b> 26 February 1999 (26.02.99)  <b>(30) Priority Data:</b> 60/076,565                      2 March 1998 (02.03.98)                      US  <b>(71) Applicant (for all designated States except US):</b> THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY OF THE DEPARTMENT HEALTH AND HUMAN SERVICES [US/US]; Centers for Disease Control and Prevention, N.E. Room 500, 255 East Paces Ferry Road, Atlanta, GA 30333 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> CARLONE, George, M. [US/US]; 5243 Sandy Shoals Lane, Stone Mountain, GA 30087 (US). ADES, Edwin, W. [US/US]; 4432 White-water Creek Road, Atlanta, GA 30327 (US). SAMPSON, Jacquelyn, S. [US/US]; 4220 Greentree Lane, College Park, GA 30349 (US). THARPE, Jean, A. [US/US]; 2669 Sherman Oaks Drive, Lithonia, GA 30058 (US). ZEILER, Joan, Louise [US/US]; 4322 N. Lockwood, Toledo, OH 43612 (US). WESTERINK, Maria, Anna, Julia [NL/US]; 8248 Country Brook Drive, Holland, OH 43528 (US).		<b>(74) Agents:</b> FOX, Robert, J. et al.; Fitch, Even, Tabin & Flannery, Room 1600, 120 South LaSalle Street, Chicago, IL 60603 (US).  <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> EPITOPE PEPTIDES IMMUNOGENIC AGAINST <i>STREPTOCOCCUS PNEUMONIAE</i>		
<b>(57) Abstract</b> <p>The invention provides a nucleic acid encoding the 37-kDa pneumococcal surface adhesion A protein (PsaA) from <i>Streptococcus pneumoniae</i>. Also provided are isolated nucleic acids comprising a unique fragment of at least 10 nucleotides of the 37-kDa protein. The invention also provides purified polypeptides encoded by the nucleic acid encoding the 37-kDa protein from and the nucleic acids comprising a unique fragment of at least 10 nucleotides of the 37-kDa protein. The invention further provides monoclonal antibodies which selectively bind PsaA. In addition peptides are provide that immunospecifically bind to the monoclonal antibodies of the invention, and that are immunogenic against <i>Streptococcus pneumoniae</i> infection. Also provided are vaccines comprising such immunogenic polypeptides, and methods of conferring protective immunity against <i>Streptococcus pneumoniae</i> infection by administering therapeutic compositions comprising the immunogenic peptides of the invention. Also provided are methods of detecting the presence of <i>Streptococcus pneumoniae</i> in a sample using antibodies or antigens, and methods of preventing and treating <i>Streptococcus pneumoniae</i> infection in a subject. In addition a method of identifying the sequence of a peptide potentially capable of eliciting protective immunity against a pathogenic microorganism is provided.</p>		

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## EPITOPE PEPTIDES IMMUNOGENIC AGAINST *STREPTOCOCCUS PNEUMONIAE*

### FIELD OF THE INVENTION

This invention relates to preventing infection by *Streptococcus pneumoniae*.  
5 More specifically, the invention relates to peptides derived from a peptide library  
that are related to the *S. pneumoniae* pneumococcal surface adhesion A protein  
(PsaA) and that are immunogenic in a subject. The invention also relates to  
pharmaceutical and therapeutic compositions containing these peptide fragments,  
and methods of conferring protection against infection by *S. pneumoniae*.

### 10 BACKGROUND OF THE INVENTION

Pneumococcal disease continues to be a leading cause of sickness and  
death in the United States and throughout the world. The currently used  
polysaccharide vaccines have limited efficacy in children under 2 years of age and  
exhibit variable serotype-specific efficacy among vaccinated individuals. For these  
15 reasons, alternative vaccine formulations have been investigated that do not require  
the use of multiple capsular polysaccharides. One current approach under  
consideration is the use of immunogenic species-common proteins as vaccine  
candidates. These proteins could be used in combination with other immunogenic  
proteins or as protein carriers in a protein, polysaccharide, or oligosaccharide  
20 conjugate vaccine. An effective vaccine that includes a common protein could  
eliminate the need for formulations based on multiple capsular polysaccharides (as  
in the current 23-valent polysaccharide vaccine) by offering a broader range of  
protection against a greater number of serotypes. Additionally, a protein-based  
vaccine would be T-cell dependent and provide a memory response, thereby  
25 resulting in a more efficacious vaccine.

An immunogenic species-common protein has been identified from  
*Streptococcus pneumoniae* (Russell *et al.* 1990, "Monoclonal antibody recognizing  
a species-specific protein from *Streptococcus pneumoniae*," J. Clin. Microbiol. 28:  
2191-2195, and U.S. Patent No. 5,422,427). A 37-kDa *S. pneumoniae* protein has  
30 been the focus of several studies and is now designated pneumococcal surface  
adhesin protein A (PsaA). (This 37-kDa protein was referred to as pneumococcal  
fimbrial protein A in U.S. Patent No. 5,422,427; the terms are used interchangeably

in the present specification.) Immunoblot analysis studies using anti-PsaA monoclonal antibody showed that PsaA is common to all 23 pneumococcal vaccine serotypes (*Russell et al.* 1990). Enzyme-linked-immunosorbent assay studies have indicated that patients with pneumococcal disease show an antibody increase in  
5 convalescent-phase serum to PsaA compared with acute-phase serum antibody levels (*Tharpe et al.* 1995, "Purification and seroreactivity of pneumococcal surface adhesin A (PsaA)," *Clin. Diagn. Lab. Immunol.* 3:227-229; and *Tharpe et al.* 1994, "The utility of a recombinant protein in an enzyme immunoassay for antibodies against *Streptococcus pneumoniae*," *Abstr. V-2*, p 617, 1994, American Society for  
10 Microbiology, Washington, D.C.). Additionally, a limited *in vivo* protection study showed that antibodies to the 37-kDa protein protect mice from lethal challenge (*Talkington et al.* 1996, "Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA)," *Microbial Pathogenesis* 21:17-22). The gene encoding PsaA from *S. pneumoniae* strain R36A  
15 (an unencapsulated strain) has been cloned in *Escherichia coli* and sequenced, but this strain does not contain a 37-kDa protein encoding nucleic acid that is highly conserved among the various serotypes. (*Sampson et al.* 1994, "Cloning and nucleotide sequence analysis of *psaA*, the *Streptococcus pneumoniae* gene encoding a 37-kilodalton protein homologous to previously reported *Streptococcus* sp.  
20 adhesins," *Infect. Immun.* 62:319-324). This particular nucleic acid and the corresponding polypeptide, therefore, are of limited value for use as diagnostic reagents, in preventing infection, in treating infection, or in vaccine development. In United States Patent Application Serial No. 08/715,131, filed Sept. 17, 1996, which is a continuation-in-part of United States Patent Application Serial No.  
25 08/222,179, filed April 4, 1994, which is a continuation-in-part of United States Patent Application Serial No. 07/791,377, filed September 17, 1991 (now U.S. Patent No. 5,422,427), all of which are hereby incorporated by reference in their entirety, an isolated nucleic acid encoding the 37-kDa protein of *Streptococcus pneumoniae*, unique fragments of at least 10 nucleotides of this nucleic acid which  
30 can be used in methods to detect the presence of *Streptococcus pneumoniae* in a sample and as immunogenic vaccines have been disclosed. Furthermore, a purified polypeptide encoded by this nucleic acid, encoding the 37-kDa protein of *Streptococcus pneumoniae*, which can be used in immunogenic vaccines, has been disclosed. Additionally, purified antibodies which bind to the 37-kDa protein of

*Streptococcus pneumoniae* or fragments thereof, which can be used in methods to detect the presence of *Streptococcus pneumoniae*, and in therapeutic and prophylactic methods, have been disclosed. Sequence conservation is a necessary requirement for a candidate species-common vaccine. The sequence conservation of the *psaA* gene among pneumococcal types, and specifically among encapsulated pneumococci which cause the vast majority of cases of serious disease, remains under investigation. There exists a need to identify characteristic epitopes related to *S. pneumoniae* PsaA in order to provide polypeptides which can serve as a vaccine for multiple strains of *Streptococcus pneumoniae*. The present invention addresses this need by determining effective epitopic peptides related to *S. pneumoniae* PsaA, and employing those peptides in therapeutic compositions directed against *Streptococcus pneumoniae* infection.

#### SUMMARY OF THE INVENTION

The present invention describes novel immunogenic peptides obtained from a random library by selecting for high affinity binding to monoclonal antibodies specific for PsaA epitopes. In addition, the peptides of the invention have the capability of serving as immunogens in a subject, thereby effectively eliciting the production of antibodies by the subject and additionally conferring protective immunity against infection by *S. pneumoniae* on the subject. The invention also relates to a selection method employed to obtain such peptides.

The invention furthermore provides a therapeutic composition in which the immunogenic peptides are combined with an immunostimulatory carrier to be administered to a subject in order to elicit an immune response which confers protective immunity against infection by *S. pneumoniae* on the subject.

The invention additionally provides a therapeutic composition in which the immunogenic peptides are combined with an adjuvant to be administered to a subject in order to elicit an immune response which confers protective immunity against infection by *S. pneumoniae* on the subject.

The invention still further describes a method of conferring protective immunity against infection by *S. pneumoniae* on a subject in which the therapeutic compositions of the invention are administered to the subject.

A further aspect of the invention presents a method for identifying a peptide incorporating PsaA or a fragment thereof (i.e., an immunogenic peptide) that elicits an immunogenic response in a subject directed against *S. pneumoniae*. The method entails preparing a random peptide library, screening the peptide  
5 library in order to identify immunogenic peptides, and obtaining the amino acid sequence of the immunogenic peptide.

The advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following  
10 detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed. Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order more fully to describe the state of the art to which this application pertains.

## 15 DETAILED DESCRIPTION OF THE INVENTION

As used herein, "immunogenic peptide" refers to a peptide which, upon being administered to a subject, or taken up by the subject in other ways, elicits an immune response. The immune response includes at least the generation of antibodies which specifically bind the immunogenic substance (i.e., a humoral  
20 response). An immunogenic substance may in addition elicit a cellular immunological response. Such an immunogen is any of the immunogenic peptides obtained by screening a library of random peptides using monoclonal antibodies that immunospecifically react with PsaA from *S. pneumoniae*.

As used herein, "immune response" and "immunogenic response" may  
25 include at least a humoral response, that is, the generation of antibodies which specifically bind the immunogenic substance. An immunogenic response may, either alternatively or in addition, refer to a cellular immunological response.

As used herein, "protective immunity" refers to a state in which a subject has generated antibodies, at least some of which are neutralizing antibodies, in  
30 response to exposure to a pathogen-related immunogen. Neutralizing antibodies bind the immunogenic component of the pathogen in such a way that proliferative infection by the pathogen is inhibited or abrogated, such that the subject remains

essentially free of symptomatic disease. Protective immunity may also arise from an alternative immunogenic response which leads to inactivation, loss, or destruction of the pathogenic agent.

As used herein, "immunostimulatory carrier" relates to any of a variety of immunogenic biological polymers which themselves elicit immune responses when introduced into a subject. Immunostimulatory carriers, when employed in conjunction with an immunogen of interest, such as the peptides of the present invention, provide enhanced immunogenic response in the subject to the immunogen of interest. Furthermore, as used herein, "adjuvant" relates to a composition that enhances the immunogenic activity of an immunogenic substance when administered in conjunction with that substance.

As used herein, a "library" refers to a set of fragments derived from a biological macromolecule, wherein each member of the set is a candidate for possessing a desired biological activity expressing a desired biological function. A library is either a peptide library or a library of oligonucleotide fragments each member of which contains a nucleotide sequence which encodes a particular member of the peptide library. In the present invention, the peptide library is a set of peptides which are coded for by a random oligonucleotide library. The desired activity for a given peptide is that the peptide be immunogenic in a subject against *S. pneumoniae*.

As used herein, a "subject" is a mammal in whom it is desired to elicit an immune response to the pathogenic organism *S. pneumoniae*. A principal class of subjects of the present invention is human beings, especially infants and elderly people, in whom *S. pneumoniae* is in fact pathogenic. In human subjects, therefore, the immune response is intended to be a protective immune response. For non-human mammals, *S. pneumoniae* may or may not be inherently pathogenic. Such non-human subjects employed as experimental animals which provide an immune response can be useful in characterizing and optimizing the compositions and methods of the invention. Such mammals include, by way of non-limiting example, mice, rats, and non-human primates. An additional class of subjects includes animals served in veterinary practice, including pets and livestock animals. If *S. pneumoniae* is pathogenic in such subjects, eliciting protective immunity is desirable.

"Purified protein" as used herein means that the protein or fragment is sufficiently free of contaminants or cell components with which the protein normally occurs as to distinguish the protein from the contaminants or cell components. It is not contemplated that "purified" necessitates having a preparation that is technically totally pure (homogeneous), but purified as used herein means the protein or polypeptide fragment is sufficiently separated from contaminants or cell components with which it normally occurs to provide the protein in a state where it can be used in an assay, such as immunoprecipitation or ELISA. For example, the purified protein can be in an electrophoretic gel.

As used herein, "stringent conditions" refers to the washing conditions used in a nucleic acid hybridization protocol. In general, the washing conditions should be a combination of temperature and salt concentration chosen so that the denaturation temperature is approximately 5-20°C below the calculated  $T_m$  of the nucleic acid hybrid under study. The temperature and salt conditions are readily determined empirically in preliminary experiments in which samples of reference DNA immobilized on filters are hybridized to the probe or protein coding nucleic acid of interest and then washed under conditions of different stringencies. The  $T_m$  of such an oligonucleotide can be estimated by allowing about 2°C for each A or T nucleotide, and about 4°C for each G or C. For example, an 18 nucleotide probe of 50% G+C would, therefore, have an estimated  $T_m$  of 54°C.

As used herein, a "therapeutic composition" relates to a composition which may be administered to a subject in order to elicit a protective immune response, and which contains one or more of the immunogenic peptides of the present invention in conjunction with an immunostimulatory carrier or an adjuvant. The therapeutic compositions contain the peptide and the carrier in either a mixture or as a chemical conjugate. Together these constitute the active agent. If more than one peptide is employed and the composition is a conjugate, each peptide is conjugated to an immunostimulatory carrier. In addition, the therapeutic composition generally contains the components of a pharmaceutical formulation in which the active agent is suspended or dissolved. The components of pharmaceutical formulations are well known to those who are skilled in immunology or pharmaceutical science. The formulation should be suitable to administer the active agent to a subject in order to elicit an immune response and



confer protective immunity against the pathogen related to the immunogenic peptide.

As used herein, the term "allelic variation" or "allelic variant" refers to an immunogenic PsaA peptide or protein obtained from a serotype of *S. pneumoniae* other than that of a reference serotype such as serotype 2. An allelic variant describes the same 37-kDa pneumococcal surface adhesin protein, or a similar protein that is diverged from the 37-kDa *Streptococcus pneumoniae* protein set forth in the Sequence Listing as SEQ ID NO:2 by less than 15% in its corresponding amino acid identity. Preferably, this allelic variant is less than 10% divergent in its corresponding amino acid identity, more preferably less than 7% divergent, more preferably less than 5% divergent, more preferably less than 3% divergent, more preferably less than 2% divergent, and most preferably less than 1% divergent in their corresponding amino acid identity. These amino acids can be substitutions within the amino acid sequence set forth in the Sequence Listing as SEQ ID NO:2, or the variants can be either deletions from or additions to the amino acid sequence set forth in the Sequence Listing as SEQ ID NO:2.

### Nucleic Acids

In one aspect, the invention provides an isolated nucleic acid encoding the 37-kDa protein of *Streptococcus pneumoniae* whose amino acid sequence is set forth in the Sequence Listing as SEQ ID NO:2. The term "isolated" refers to a nucleic acid which is essentially separated from other genes that naturally occur in *S. pneumoniae*. In one embodiment, the present invention provides an isolated nucleic acid encoding the 37-kDa protein of *Streptococcus pneumoniae* wherein the nucleic acid is the nucleic acid whose nucleotide sequence is set forth in the Sequence Listing as SEQ ID NO:1. An isolated nucleic acid comprising a unique fragment of at least 10 nucleotides of the nucleic acid set forth in the Sequence Listing as SEQ ID NO:1 is also provided. "Unique fragments," as used herein, means a nucleic acid of at least 10 nucleotides that is not identical to any other known nucleic acid sequence at the time the invention was made. Examples of the sequences of at least 10 nucleotides that are unique to the nucleic acid set forth in the Sequence Listing as SEQ ID NO: 1 can be readily ascertained by comparing the sequence of the nucleic acid in question to sequences catalogued in GenBank, or other sequence database, using computer programs such as DNASIS (Hitachi

engineering, inc.), or Word Search or FASTA of the Genetics Computer Group (GCG) (Madison, WI), which search the catalogued nucleotide sequences for similarities to the nucleic acid in question. If the sequence does not match any of the known sequences, it is unique. For example, the sequence of nucleotides 1-10  
5 can be used to search the databases for an identical match. If no matches are found, then nucleotides 1-10 represent a unique fragment. Next, the sequence of nucleotides 2-11 can be used to search the databases, then the sequence of nucleotides 3-12, and so on up to nucleotides 1321 to 1330 of the sequence set forth in the Sequence Listing as SEQ ID NO:1. The same type of search can be  
10 performed for sequences of 11 nucleotides, 12 nucleotides, 13 nucleotides, etc. The possible fragments range from 10 nucleotides in length to 1 nucleotide less than the sequence set forth in the Sequence Listing as SEQ ID NO:1. These unique nucleic acids, as well as degenerate nucleic acids can be used, for example, as primers for amplifying nucleic acids from other strains of *Streptococcus*  
15 *pneumoniae* in order to isolate allelic variants of the 37-kDa protein, or as primers for reverse transcription of 37-kDa protein RNA, or as probes for use in detection techniques such as nucleic acid hybridization. One skilled in the art will appreciate that even though a nucleic acid of at least 10 nucleotides is unique to a specific gene, that nucleic acid fragment can still hybridize to many other nucleic acids and  
20 therefore be used in techniques such as amplification and nucleic acid detection.

Also provided are nucleic acids which encode allelic variants of the 37-kDa protein of *S. pneumoniae* set forth in the Sequence Listing as SEQ ID NO:2. The homology between the protein coding region of the nucleic acid encoding the allelic variant of the 37-kDa protein is preferably less than 20% divergent from the  
25 region of the nucleic acid set forth in the Sequence Listing as SEQ ID NO:1 encoding the 37-kDa protein. Preferably, the corresponding nucleic acids are less than 15% divergent in their sequence identity. In another embodiment, the corresponding nucleic acids are less than 10% divergent in their sequence identity, more preferably less than 7% divergent, more preferably less than 5% divergent,  
30 more preferably less than 4% divergent, more preferably less than 3% divergent, more preferably less than 2% divergent, and most preferably less than 1% divergent in their corresponding nucleotide identity. In particular, the nucleic acid variations can create up to about 15% amino acid sequence variation from the protein set forth in the Sequence Listing as SEQ ID NO:2.

One skilled in the art will appreciate that nucleic acids encoding homologs or allelic variants of the 37-kDa protein set forth in the Sequence Listing as SEQ ID NO:2 can be isolated from related gram-positive bacteria. The nucleic acid encoding a 37-kDa protein may be obtained by any number of techniques known to one skilled in the art. Methods of isolating nucleic acids of the invention, including probes and primers that may be used, are set forth in United States Patent Application Serial No. 08/715,131, filed Sept. 17, 1996, which is a continuation-in-part of United States Patent Application Serial No. 08/222,179, filed April 4, 1994, which is a continuation-in-part of United States Patent Application Serial No. 07/791,377, filed September 17, 1991 (now U.S. Patent No. 5,422,427). General methods that may be employed for these purposes are set forth in Sambrook *et al.*, "Molecular Cloning, a Laboratory Manual," Cold Spring Harbor Laboratory Press (1989), and Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley and Sons, New York 1987 (updated quarterly). Amplification procedures that may be employed in the nucleic acid isolation protocols are well known to those skilled in the art (see, for example, Innis *et al.* 1990 "PCR Protocols: A Guide to Methods and Applications" Academic Press, Inc.). An example of amplification of a nucleic acid encoding the 37-kDa protein of *Streptococcus pneumoniae* serotype 6B is discussed in the Example contained herein.

### 37-kDa Protein

The present invention also provides a purified polypeptide as set forth in the Sequence Listing as SEQ ID NO:2 and a purified polypeptide encoded by a nucleic acid comprising a unique fragment of at least 10 nucleotides of SEQ ID NO:1. The protein can be used as a vaccine component as well as a reagent for identifying subject antibodies raised against *Streptococcus pneumoniae* during infection. The purified protein can also be used in methods for detecting the presence of *Streptococcus pneumoniae*.

Unique fragments of the 37-kDa protein can be identified in the same manner as that used to identify unique nucleic acids. For example, a sequence of 3 amino acids or more, derived from the sequence of the 37-kDa protein, as set forth in the Sequence Listing as SEQ ID NO:2, can be used to search the protein sequence databases. Those that do not match a known sequence are therefore unique. Methods of preparing these proteins and protein fragments are set forth in

United States Patent Application Serial No. 08/715,131, filed Sept. 17, 1996, which is a continuation-in-part of United States Patent Application Serial No. 08/222,179, filed April 4, 1994, which is a continuation-in-part of United States Patent Application Serial No. 07/791,377, filed September 17, 1991 (now U.S. Patent No. 5,422,427).

The present invention provides peptide fragments related to the 37-kDa pneumococcal surface adhesin protein. The polypeptide fragments of the present invention can be recombinant polypeptides obtained by cloning nucleic acids encoding fragments of the polypeptide in an expression system capable of producing the polypeptide fragments thereof, as described above for the 37-kDa protein. For example, one can identify an immunoreactive peptide related to the 37-kDa pneumococcal surface adhesin protein which can cause a significant immune response by using antibodies raised against the adhesin protein, cloning the nucleic acid encoding that polypeptide into an expression vector, and isolating that particular polypeptide for further uses, such as diagnostics, therapy, and vaccination. Amino acids which do not contribute to the immunoreactivity and/or specificity can be deleted without a loss in the respective activity. For example, amino or carboxy-terminal amino acids can be sequentially removed from any peptide identified using the procedure outlined above, and the immunoreactivity tested in one of many available assays. Alternatively, internal amino acids can be sequentially removed and the immunoreactivity tested for each of the deletions.

In another example, a peptide fragment related to a 37-kDa pneumococcal surface adhesin protein can comprise a modified polypeptide wherein at least one amino acid has been substituted for the amino acid residue originally occupying a specific position, or a portion of either amino terminal or carboxy terminal amino acids, or even an internal region of the polypeptide, can be replaced with a polypeptide fragment or other moiety, such as biotin, which can facilitate in the purification of the modified 37-kDa pneumococcal surface adhesin protein.

Immunoreactive peptide fragments related to a 37-kDa pneumococcal surface adhesin protein can include insertions, deletions, substitutions, or other selected modifications of particular regions or specific amino acids residues, provided the immunoreactivity of the peptide is not significantly impaired compared to the 37-kDa pneumococcal surface adhesin protein. These modifications can provide for some additional property, such as to remove/add

amino acids capable of disulfide bonding, to increase its bio-longevity, and the like. In any case, the peptide must possess a bioactive property, such as immuno-reactivity, comparable to the 37-kDa pneumococcal surface adhesin protein.

### Antibodies

5           The present invention employs a purified antibody which selectively binds with the polypeptide encoded by the nucleic acid set forth in the sequence listing as SEQ ID NO. 1, or a polypeptide encoded by a unique fragment of at least 10 nucleotides of SEQ ID NO: 1. The antibody (either polyclonal or monoclonal) can be raised to the 37-kDa pneumococcal surface adhesin protein or a unique fragment thereof, in its naturally occurring form or in its recombinant form. The antibody can be used in a variety of techniques or procedures such as diagnostics, treatment, or immunization. Antibodies can be prepared by many well-known methods (see, e.g. *Harlow and Lane*, "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1988)). Briefly, purified antigen can be injected into an animal in amount and at intervals sufficient to elicit an immune response. Antibodies can be purified directly, to yield polyclonal antibodies. Alternatively, spleen cells can be obtained from the animal. The cells can then fused with an immortal cell line and screened for antibody secretion to yield monoclonal antibodies. The antibodies can be used to screen nucleic acid clone libraries for cells secreting the antigen. Those positive clones can then be sequenced (see, for example, *Kelly et al.*, *Bio Technology*, 1992 10:163-167; *Bebbington et al.*, 1992 *Bio Technology*, 10:169-175).

20           The phrase "selectively binds" with the polypeptide refers to a binding reaction which is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bound to a particular protein do not bind in a significant amount to other proteins present in the sample. Selective binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats may be used to select antibodies which selectively bind with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies selectively immunoreactive with a protein. See *Harlow and Lane* "Antibodies: A Laboratory Manual" Cold Spring Harbor Publications, New York, (1988), for a description of

immunoassay formats and conditions that could be used to determine selective binding. In some instances, it is desirable to prepare monoclonal antibodies from various subjects. A description of techniques for preparing such monoclonal antibodies may be found in *Stites et al.*, editors, "Basic and Clinical Immunology," (Lange Medical Publications, Los Altos, Calif, Fourth Edition) and references cited therein, and in *Harlow and Lane* ("Antibodies: A Laboratory Manual" Cold Spring Harbor Publications, New York, (1988)).

The monoclonal antibodies (MAbs) employed in the present invention (disclosed in United States Patent Application Serial No. 08/715,131, filed Sept. 17, 1996, incorporated herein by reference) are MAb 1E7A3D7C2, or a fragment thereof which retains the characteristics of antibody 1E7A3D7C2, such as its binding specificity and its binding affinity; MAb 1B6E12H9, or a fragment thereof which retains the characteristics of antibody 1B6E12H9; MAb 3C4D5C7, or a fragment thereof which retains the characteristics of antibody 3C4D5C7; MAb 4E9G9D3, or a fragment thereof which retains the characteristics of antibody 4E9G9D3; MAb 4H5C10F3, or a fragment thereof which retains the characteristics of antibody 4H5C10F3; MAb 6F6F9C8, or a fragment thereof which retains the characteristics of antibody 6F6F9C8; and MAb 8G12G11B10, or a fragment thereof which retains the characteristics of antibody 8G12G11B10.

The hybridomas used to produce the respective monoclonal antibodies employed in the present invention (disclosed in United States Patent Application Serial No. 08/715,131, filed Sept. 17, 1996, incorporated herein by reference) are hybridoma 1E7A3D7C2, hybridoma 1B6E12H9, hybridoma 3C4D5C7, hybridoma 4E9G9D3, hybridoma 4H5C10F3, hybridoma 6F6F9C8, and hybridoma 8G12G11B10.

### **Therapeutic Compositions**

Also provided by the present invention is a therapeutic composition comprising an immunogenic polypeptide encoded by the nucleic acid as set forth in the Sequence Listing as SEQ ID NO:1, or a unique fragment of at least 10 nucleotides of SEQ ID NO:1. The invention also provides therapeutic compositions comprising at least one immunogenic polypeptide that immunospecifically binds to a monoclonal antibody obtained in response to immunizing an animal with *Streptococcus pneumoniae* PsA. The therapeutic

composition is preferably combined with an immunostimulatory carrier. The therapeutic composition confers protective immunity against *S. pneumoniae* infection when administered to a subject.

The polypeptides provided by the present invention can be used to  
5 vaccinate a subject for protection from a particular disease, infection, or condition caused by the organism from which the 37-kDa pneumococcal surface adhesin protein (or a unique fragment thereof) was derived. Polypeptides of a 37-kDa pneumococcal surface adhesin protein of serotype 6B, or a unique fragment thereof, can be used to inoculate a subject organism such that the subject generates an  
10 active immune response to the presence of the polypeptide or polypeptide fragment which can later protect the subject from infection by organism from which the polypeptide was derived. One skilled in the art will appreciate that an immune response, especially a cell-mediated immune response, to a 37-kDa pneumococcal surface adhesin protein from a specific strain can provide later protection from  
15 reinfection or from infection from a closely related strain. The 37-kDa protein provided by the present invention, however, is relatively conserved among the 90 serotypes of *S. pneumoniae* and can, therefore, serve as a multivalent vaccine. Immunization with the 37-kDa pneumococcal surface adhesin protein or with the immunogenic peptides of the invention can be achieved by administering to  
20 subjects the 37-kDa pneumococcal surface adhesin protein either alone or with a pharmaceutically acceptable carrier. (Kuby, J. 1992 "Immunology," W.H. Freeman and Co. New York). Immunogenic amounts of the 37-kDa pneumococcal surface adhesin protein or of the immunogenic peptides of the invention can be determined using standard procedures. Briefly, various concentrations of the present  
25 polypeptide are prepared, administered to subjects, and the immunogenic response (e.g., the production of antibodies to the polypeptide or cell mediated immunity) to each concentration is determined. Techniques for monitoring the immunogenic response, both cellular and humoral, of patients after inoculation with the polypeptide, are well known in the art. For example, samples can be assayed using  
30 enzyme-linked immunosorbent assays (ELISA) to detect the presence of specific antibodies, such as serum IgG (Hjelt *et al.* J. Med. Virol. 21:39-47, (1987)); lymphocyte or cytokine production can also be monitored. The specificity of a putative immunogenic antigen of any particular polypeptide can be ascertained by testing sera, other fluids, or lymphocytes from the inoculated patient for cross-

reactivity with other closely related 37-kDa pneumococcal surface adhesin proteins. The amount of a polypeptide of the 37-kDa pneumococcal surface adhesin protein or of the immunogenic peptides of the invention to be administered will depend on the subject, the condition of the subject, the size of the subject, and the like, but will be at least an immunogenic amount. The polypeptide can be formulated with adjuvants and with additional compounds, including cytokines, with a pharmaceutically acceptable carrier.

The pharmaceutically acceptable carrier or adjuvant in the therapeutic composition of the present invention can be selected by standard criteria (*Arnon, R.* (Ed.) "Synthetic Vaccines" 1:83-92, CRC Press, Inc., Boca Raton, Florida, 1987). By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable (i.e., the material may be administered to an individual along with the selected compound without causing any undesirable biological effects or interacting in an undesirable manner with any of the other components of the pharmaceutical composition in which it is contained). The carrier or adjuvant may depend on the method of administration and the particular patient. Methods of administration can be parenteral, oral, sublingual, mucosal, inhaled, absorbed, or injection. Actual methods of preparing the appropriate dosage forms are known, or will be apparent, to those skilled in this art; see, for example, *Remington's Pharmaceutical Sciences* (Martin, E.W. (ed.) latest edition Mack Publishing Co., Easton, PA). Parenteral administration, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Another approach for parenteral administration involves use of a slow release or sustained release system, such that a constant level of dosage is maintained (see, e.g., U.S. Patent No 3,710,795). In addition, powders or aerosols may be formulated for administration by inhalation.

### Detection Methods

The present invention provides methods of detecting the presence of *Streptococcus pneumoniae* in a subject, based on several variations of immunoassays, using either a purified polypeptide encoded by the nucleic acid set forth in the Sequence Listing as SEQ ID NO:1, a purified polypeptide encoded by a nucleic acid comprising a unique fragment of at least 10 nucleotides of SEQ ID



NO:1, an antibody which selectively binds the purified polypeptide encoded by the nucleic acid set forth in the Sequence Listing as SEQ ID NO:1, or an antibody which selectively binds a purified polypeptide encoded by a nucleic acid comprising a unique fragment of at least 10 nucleotides of SEQ ID NO:1, and detecting the binding of the antibody with the polypeptide, the binding indicating the presence of *Streptococcus pneumoniae* in the subject. There are numerous immunodiagnostic methods that can be used to detect antigen or antibody as the following non-inclusive examples illustrate. These methods, as well as others, can not only detect the presence of antigen or antibody, but quantitate antigen or antibody as well. These methods are set forth in United States Patent Application Serial No. 08/715,131, filed Sept. 17, 1996, which is a continuation-in-part of United States Patent Application Serial No. 08/222,179, filed April 4, 1994, which is a continuation-in-part of United States Patent Application Serial No. 07/791,377, filed September 17, 1991 (now U.S. Patent No. 5,422,427). In general, the detection methods that may be employed in practicing the present invention are described in, for example, *Harlow et al.*, "Antibodies: A Laboratory Manual" Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1988).

#### Methods of Treating and Preventing Infection

The present invention also provides a method of preventing *Streptococcus pneumoniae* infection in a subject at risk of infection by *S. pneumoniae*, comprising administering to the subject an effective amount of a therapeutic composition comprising an immunogenic polypeptide encoded by the nucleic acid encoding the 37-kDa protein of *Streptococcus pneumoniae* as set forth in the Sequence Listing as SEQ ID NO:1, or an immunogenic polypeptide encoded by a nucleic acid comprising a unique fragment of at least 10 nucleotides of SEQ ID NO:1, or the immunogenic peptides of the invention either alone or with a pharmaceutically acceptable carrier.

The present invention further provides a method of treating a *Streptococcus pneumoniae* infection in a subject, comprising administering to the subject an effective amount of an antibody to the polypeptide encoded by the nucleic acid set forth in the Sequence Listing as SEQ ID NO:1, or a polypeptide encoded by a nucleic acid comprising a unique fragment of at least 10 nucleotides of SEQ ID NO:1, either alone or with a pharmaceutically acceptable carrier. Treating a

subject already infected with a particular organism by administering to the subject an antibody against the organism is well known in the art. For example, immune globulin isolated from animals or humans previously exposed to rabies virus is currently a therapy for rabies virus infection. Better treatment of infected individuals can be achieved by administering to those individuals monoclonal antibodies since those monoclonals react or bind more specifically than the polyclonals. (See, e.g. *Kaplan et al.* "Rabies" *Sci. Am.* 242:120-134 (1980)).

### Epitopic Immunogenic Peptides

The present invention discloses novel epitopic immunogenic peptides obtained as the peptides coded in a random oligonucleotide library by selecting for high affinity binding of the epitopes to monoclonal antibodies specific for epitopes on the PsaA antigen.

In an additional method, a procedure known as "biopanning" or "panning", a target protein or peptide is selected from a library expressed as a heterologous insert on an external surface of a microorganism. A bacterium or virus, for example, may have a nucleotide sequence encoding a heterologous peptide or protein sequence incorporated into its chromosomal nucleic acid in such a way that a fusion or chimera is created. The fusion represents a natural protein of the microorganism directly linked with the heterologous peptide or protein. Once expressed on the surface of the microorganism, it can be probed by a ligand specific for the sought peptide or protein, such as an antibody. Once identified by capture, the heterologous sequence, either the nucleic acid or the protein, can be obtained and identified.

A common implementation of this procedure is well-known to those of skill in the fields of protein chemistry, immunology, and virology. A filamentous bacteriophage such as M13, f1, or fd is employed. These bacteriophages have two well-known structural proteins on their surfaces: the gene III protein and the gene VIII protein. The nucleic acid of the phage is altered by incorporating a fusion sequence of the heterologous peptide in frame with the gene for one or the other of these structural proteins. When one is seeking a target peptide from among a large set, or library, of such peptides, the corresponding library of heterologous nucleotide sequences coding for the members of the peptide library is incorporated into the structural protein gene. The resulting bacteriophage population (termed a

phage display library) is subjected to procedures which optimize selection of only those virus particles expressing members of the peptide library for which the PsaA-specific ligand, such as an MAb, has a high affinity. The bacteriophage particles so selected may then be amplified by further culture, or their nucleic acids may be amplified by methods such as polymerase chain reaction. In this way the nucleic acid of the captured particle may be isolated and sequenced to provide the coding sequence for the high affinity epitope bound to the MAb or other ligand.

Biopanning is described for example, in Smith, G.P. and K.K. Scott (1993, "Libraries of Peptides and Proteins Displayed on Filamentous Phage", Meth. Enzymol. 217: 228-257).

The immunogenic peptides of the invention were obtained using a biopanning procedure that has general applicability for identifying the sequence of a peptide potentially capable of eliciting protective immunity against a pathogenic microorganism. The method includes the steps of

(a) providing a library comprised of random oligonucleotides, wherein the oligonucleotides are about 30-45 nucleotides in length;

(b) splicing the oligonucleotides of a library into the gene for a coat protein of a filamentous bacteriophage in frame with the codons for the amino acid residues of the coat protein, such that the gene for the coat protein is contained within the complete nucleic acid that is the genome for the bacteriophage, thereby creating a bacteriophage library, and further positioning the oligonucleotides within the gene such that when the coat protein is expressed and incorporated into a complete bacteriophage particle the peptide is available, by exposure on the surface, as an epitope to which an antibody can bind;

(c) expanding the bacteriophage library harboring the oligonucleotide library by culturing the bacteriophage library in a host which the bacteriophage infects;

(d) screening the expanded bacteriophage library for any bacteriophage particle that immunospecifically reacts with a monoclonal antibody obtained in response to immunizing an animal with an immunogen of the microorganism; and

(e) sequencing the gene for the coat protein of any bacteriophage particle obtained in step (d) thereby yielding the nucleotide sequence of that member of the oligonucleotide library whose translation product has the sequence of a peptide potentially capable of eliciting protective immunity against *Streptococcus*

*pneumoniae*.

In the particular application employed in obtaining the immunogenic peptides of the invention, the method described above is directed against *S. pneumoniae*, the coat protein is the gene III protein which is the tail protein of a filamentous bacteriophage such as M13, f1, or fd, and the monoclonal antibody is obtained in response to immunizing an animal with *Streptococcus pneumoniae* pneumococcal surface adhesion A protein (PsaA). The peptides are isolated using a procedure that emphasizes capturing only those peptides that have a high affinity for the antibodies. This assures that any protective effect based on humoral immunity will be highly effective

The sequences of the peptides which bind to the antibodies may be identified by sequencing the gene III fusion of the bacteriophage particle obtained in the biopanning process. The actual immunogenic peptides may then be synthesized in conventional peptide synthesizers. These peptides are then incorporated into a therapeutic composition in which the immunogenic peptides are combined with an immunostimulatory carrier to be administered to a subject. Upon being administered in effective amounts, the subject elicits the production of antibodies against *S. pneumoniae*. This results in conferring protective immunity against infection by *S. pneumoniae* on the subject.

PsaA is a 37-kDa species-common protein from *S. pneumoniae* (pneumococcus) which is effectively immunogenic. It is common to all the serotypes whose polysaccharides are components of the pneumococcal vaccine currently in use (Russell *et al.*, 1990, "Monoclonal antibody recognizing a species-specific protein from *Streptococcus pneumoniae*", J. Clin. Microbiol. 28: 2191-2195). The sequence of the *PsaA* gene cloned from serotype R36A has been described (U. S. Patent 5,422,427, to Russell *et al.*), and the sequence of PsaA protein was deduced. In addition, the nucleotide sequence of cloned *PsaA* from serotypes 2 and 6B, and their corresponding amino acid sequences, have been determined (Berry *et al.*, 1996, "Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*", Infect. Immun. 64: 5255-5262; Sampson *et al.*, 1997, "Limited Diversity of *Streptococcus pneumoniae* *psaA* among Pneumococcal Vaccine Serotypes", Infect. Immun. 65: 1967-1971). Excluding the putative leader sequence, there are 6 amino acid differences between PsaA's from serotype 6B versus serotype 2, out of a total of

290 residues overall; there are 45 amino acid differences between 6B and 36A (Sampson et al., *ibid*). This result led Sampson et al. to suggest that serotypes 2 and 6B represent the prototypical sequences among pneumococcal PsaA proteins. PsaA from serotype 3 (disclosed in United States Patent Application Ser. No. 08/715,131, incorporated herein by reference) and serotype 22 (Talkington et al., 1996, "Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA)", Microb. Pathog. 21: 17-22) effectively provide protective immunity in mice against challenge doses of *S. pneumoniae*.

The peptides of the present invention contain immunogenic epitopes selected by binding to PsaA-specific monoclonal antibodies. Preferably the peptide is about 10-25 residues in length. More preferably, the peptide is about 12-22 residues in length, and most preferably about 15 residues in length. In the embodiments presented in the Examples below, the peptides are given in SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8. In addition, the invention encompasses immunogenic peptides which may be shorter than these sequences. Thus, for example, immunogenic fragments of SEQ ID NO:5, immunogenic fragments of SEQ ID NO:6, immunogenic fragments of SEQ ID NO:7, and immunogenic fragments of SEQ ID NO:8 are also encompassed by the present invention.

Currently approximately 90 serotypes of *S. pneumoniae* have been identified; these may have PsaA antigens which are allelic variants of the PsaA sequences already identified. The invention therefore encompasses an allelic immunogenic peptide which, for example, was obtained by a biopanning procedure in which the monoclonal antibodies were raised by immunizing with an allelic variant, or in other ways known to those skilled in the relevant arts. The sequence of such a peptide is at least 80% identical to any of the following sequences: SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, immunogenic fragments of SEQ ID NO:5, immunogenic fragments of SEQ ID NO:6, immunogenic fragments of SEQ ID NO:7, and immunogenic fragments of SEQ ID NO:8.

The monoclonal antibodies (MAbs) disclosed above were used further in procedures of the present invention. The specific MAbs that were used are designated 1E7 (1E7A3D7C2), 6F6 (6F6F9C8), 4E9 (4E9G9D3), 8G12 (8G12G11B10), and 1B6 (1B6E12H9). These MAbs were obtained as a result of

immunization of an animal with PsaA: such antibodies therefore represent molecules whose antigen-binding domains bind immunogenic epitopes of the invention.

5 Identification of immunogenic epitopes related to PsaA may be achieved in any of a number of ways. Methods to identify immunogenic epitopes may employ any MAb obtained in response to primary immunization with PsaA. Any procedure which narrows down the overall molecular structure of PsaA to moieties or fragments thereof may be employed in identifying immunogenic epitopes thereof. In one method, chemical modification of specific residues of PsaA yields  
10 modified products whose reactivity with a ligand such as an anti-PsaA MAb may be impaired. Knowledge of which residue or residues were modified in products with impaired binding may be used to identify those residues as potentially being a portion of the epitope. Additionally, biopanning, described above, may be used.

In another method, fragments of PsaA may be synthesized chemically by  
15 peptide synthesis. In general, a set of peptides are synthesized which represents a systematic progression along the entire sequence of the protein from its N-terminus to its C-terminus. Windows of predetermined lengths may be "walked" along the protein sequence generating a set of peptides which encompasses most or all of the original sequence. Methods of peptide synthesis are well-known to workers of skill  
20 in the fields of peptide chemistry, protein chemistry, and immunology. Commercial instruments are available for the automated synthesis of peptides once their sequences are specified. A set of peptides obtained in this way may be subjected to assays which establish whether they bind to PsaA-specific ligands, such as anti-PsaA MAbs. Immunoassay methods are preferred for such  
25 determinations, and are well-known to workers of skill in immunology. They include procedures such as enzyme-linked immunosorbent assays (ELISA), using, for example, competitive formats or direct heterogeneous formats. Peptides found to bind with high affinity to the PsaA-specific ligands are presumed to contain or encompass an immunogenic epitope of PsaA.

30 The immunogenic peptides of the invention are identified in the selection or screening procedures described in the preceding paragraphs. The sequences of the peptides positively selected next need to be obtained. In the case of chemical modification, the location of inhibitory modifications in the sequence yields peptides centered on, or containing, that modified residue. In the case of the

screening of synthesized peptides, the sequence is immediately available from the identity of the positive sample. In the case of biopanning, the positive bacteriophages are isolated and the nucleic acid is amplified, either by expansion of the phage particles in culture or by amplification of the nucleic acid itself. The nucleic acid is then isolated and sequenced to identify the coding sequence for the heterologous peptide and the coding sequence translated to yield the peptide sequence.

Once the sequences are known, the corresponding peptides are synthesized in order to serve as immunogenic peptides in a subject. In general, the peptides will be combined with an immunostimulatory carrier and/or with an adjuvant prior to being administered to a subject. In common practice, immunostimulatory carriers are proteins such as keyhole limpet hemocyanin, bovine serum albumin, thyroglobulin, diphtheria toxoid, and the like. The immunogenic peptides and the carrier may be combined either noncovalently or covalently. When combined noncovalently, they are mixed together so that they comprise components in a therapeutic composition to be administered to a subject. An adjuvant useful in the such a composition, by way of nonlimiting example, is alum. When covalently combined, the immunogen is conjugated with the immunostimulatory carrier using chemical reagents and chemical procedures well known to workers of skill in the fields of protein chemistry and immunology. If a mixture of immunogenic peptides is employed, each is conjugated to an immunostimulatory carrier. In the present invention, it is preferred to employ conjugated adducts of the immunogenic peptide with the carrier.

In preparing the therapeutic composition of the invention, the combination of the immunogenic peptide and the immunostimulatory carrier is formulated with a pharmaceutically acceptable vehicle for administration to a subject. As already described, such vehicles are well known to those of skill in the pharmaceutical sciences, and include preparations in liquid, gel, or solid forms, for administration by oral, sublingual, mucosal, and parenteral routes, including inhalation. These dosage forms may be conventional preparations such as solutions or suspensions having immediate bioavailability, or they may be controlled release formulations or devices having the property of releasing the active immunogenic peptide slowly over an extended time period. The therapeutic composition confers protective immunity against *S. pneumoniae* in a subject to whom it is administered.

In addition to peptides discovered by the methods herein described, immunogenic fragments of such peptides are also encompassed within the present invention. An immunogenic fragment is any peptide shorter than the peptide from which it is derived (the parent) whose sequence is identical to the sequence of a portion of the parent peptide and which retains immunogenicity. It is generally understood in the field of immunochemistry that such peptides must be at least about six residues long in order to be antigenic. Thus any fragment should be at least six residues in length and may have a maximum length one residue less than the parent peptide. Identifying immunogenic fragments can be accomplished using any method which will identify immunogenicity. These methods include, for example, the biopanning procedure described above, as well as direct demonstration of immunogenicity by combining the candidate peptide with an immunostimulatory carrier to form the active component of a pharmaceutical composition, administering the pharmaceutical composition to a subject and assessing whether an immunogenic response has occurred.

A peptide fragment which has been positively identified as being immunogenic may also be assessed for its ability to elicit protective immunity in a subject. This is carried out using methods described herein for determining whether an experimental subject animal exhibiting an immunogenic response to a PsaA peptide fragment resists a challenge by *S. pneumoniae*.

In addition to therapeutic compositions in which the active agent is a single immunogenic peptide of the invention, the compositions may also include active agents constituted to contain mixtures of peptides having the sequences given by SEQ ID NO:5 or an immunogenic fragment thereof, SEQ ID NO:6 or an immunogenic fragment thereof, SEQ ID NO:7 or an immunogenic fragment thereof, SEQ ID NO:8 or an immunogenic fragment thereof, or a fragment of SEQ ID NO:2 whose length is 10-25 residues, preferably 12-22 residues, or more preferably about 15 residues.

Additional peptides which are immunogenic and comprise the active agent in therapeutic compositions of the invention are peptides containing an immunogenic peptide related to an allelic variant of PsaA. Such peptides are obtained by a procedure in which monoclonal antibodies were raised by immunizing with an allelic variant, and are at least 80%, preferably at least 90%, and most preferably at least 95%, identical to peptides whose sequences have been



set forth above.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of the present invention. They are intended to be purely exemplary of the invention and not to limit the scope of what the inventors regard as their invention. Unless indicated otherwise, parts are parts by weight, temperature is in °C, and pressure is at or near atmospheric.

### EXAMPLES

Bacterial strains The *S. pneumoniae* strain R36A was kindly provided by D E Briles (University of Alabama at Birmingham). Twenty-four serotypes of *S. pneumoniae* were provided by K. Facklam, Centers for Disease Control (CDC), Atlanta, Ga. These serotypes are 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11F, 11A, 12F, 14, 15B, 18C, 19A, 19F, 20, 22F, 23F, and 33F. *Enterococcus avium*, *E. casseliflavus*, and *E. gallinarum* were also provided by R. Facklam. Anaerobic bacteria were obtained from V.R. Lowell, CDC. These included *Bacteroides asaccharolyticus*, *B. fragilis*, *B. intermedius*, *B. thetaiotaomicron*, *Eubacterium lentum*, *Fusobacterium necrophorum*, *F. nucleatum*, *Peptostreptococcus anaerobius*, *P. asaccharolyticus*, *Propionibacterium acnes*, and *Staphylococcus saccharolyticus*. *Branhamella catarrhalis* and *Bordetella parapertussis* were obtained from R. Weaver, CDC. *Mycobacterium tuberculosis* was provided by R.C. Good, CDC. R. Barnes, CDC, provided *Chlamydia pneumoniae*. The following remaining bacteria were from the stock collection of the Immunology Laboratory, CDC: *Bordetella pertussis*, *Enterobacter aerogenes*, *E. agglomerans*, *E. cloacae*, *E. gergoviae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Haemophilus influenzae* (types a-f), *Legionella micdadei*, *L. pneumophila*, *Mycoplasma pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *S. equisimilis*, *S. pyogenes*, and group G streptococci.

Production of MAbs Female BALB/c mice were immunized with whole cell suspensions of *S. pneumoniae* R36A, a rough derivative of the capsular type 2 strain D39 (Avery et al. (1944) J. Exp. Med. 79:137-157). The mice were immunized by intravenous injection three times and once by intraperitoneal injection. The maximum number of cells injected at any time was about  $10^8$ .

Fusion was done on day 25 by using standard procedures (*Clafin et al.* (1978) Curr. Top. Microbiol. Immunol. 81:107-109). Spleen cells of 4 mice were fused with Sp2/0-Ag14 myeloma cells (*Schulman et al.* (1978) Nature (London) 276:269-270). Culture fluids of the growing hybridomas were tested for antibodies to *S. pneumoniae* whole cells in an ELISA. A clone designated 1E7A3D7C2 was one of 10 selected for further study.

ELISA. Screening of hybridoma culture supernatants was done by ELISA. U-bottom microtitration plates (Costar, Cambridge, Mass.) were sensitized with 50  $\mu$ l of *S. pneumoniae* whole cell suspension ( $10^9$  CFU/ml) diluted 1:4,000 in 0.1 M carbonate buffer, pH 9.6, and kept for 16 h at 4°C. The plates were washed 5 times with 0.9% NaCl containing 0.05% Tween 20 (NaCl-T). Culture supernatants (50  $\mu$ l) from the fusion plates were added to 50  $\mu$ l of a solution containing 2% bovine serum albumin (BSA), 10% normal rabbit serum, 0.3% Tween-20, and 0.02% Merthiolate in phosphate buffered saline (PBS), pH 7.2, (ELISA diluent; *Wells et al.* (1987) J. Clin. Microbiol. 25:516-521) in the plates and were incubated for 30 min at 37°C. The plates were washed 5 times with NaCl-T. Fifty microliters of goat anti-mouse immunoglobulin horseradish peroxidase conjugate in ELISA diluent was added to each well. The plates were incubated for 30 min at 37°C. The plates were washed, and 50  $\mu$ l of 3,3',5,5'-tetramethylbenzidine (0.1 mg/ml in 0.1M sodium acetate, 0.1 M citric acid (pH 5.7) with 0.005% hydrogen peroxide) was added to each well and incubated for 30 min at 37°C. The reaction was stopped by adding 1 ml of 4 M H<sub>2</sub>SO<sub>4</sub> and the optical density was read on a Dynatech ELISA Reader (Dynatech Laboratories, Inc., Alexandria, Va.) at 450 nm. An optical density of greater than 0.200 was considered positive.

SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Tsang et al. ((1983) Methods Enzymol. 92:377-391), using an 8% acrylamide resolving gel. Equal volumes of sample buffer (5% SDS-10% 2-mercaptoethanol-20% glycerol in 0.01 M Tris HCl, pH 8.0) and cell suspension containing 2.4  $\mu$ g protein per  $\mu$ l were mixed, heated at 100°C for 5 min, and a 5- $\mu$ l sample was applied to 1 of 15 wells. If the final protein content of the portion of sample to be tested was <1.2  $\mu$ g/ $\mu$ l, a volume up to 10  $\mu$ l of sample was applied to achieve a final concentration of 6  $\mu$ l of protein per well. Protein concentrations were determined by the method of Markwell et al. ((1978) Anal. Biochem. 87:206-210).

with BSA as the standard. Proteins separated by SDS-PAGE were either silver stained by the method of Morrissey ((1981) Anal. Biochem. 117:307-310) or electroblotted onto nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.). The immunoblot procedure was done according to the method of Tsang et al. (1983) with slight modifications. The blots were given three 5-min washes with PBS, pH 7.2, containing 0.3% Tween-20 and were gently agitated overnight (16 h) at 25°C. The blots were blocked for 1 h with casein-thimerosal buffer (CTB) (*Kenna et al.* (1985) J. Immunol. Meth. 85:409-419). After three rinses with CTB, the blots were exposed to goat anti-mouse immunoglobulin horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif) for 2 h at 25°C. Conjugate dilutions (1:2,000) were made in CTB. The blots were again rinsed three times with CTB and exposed to 3,3'-diaminobenzidine-4-hydrochloride in PBS, pH 7.2 (0.5 mg/ml), with 0.003% H<sub>2</sub>O<sub>2</sub> for 5 min at 25°C. Reactivity was expressed as a visible colored band on the nitrocellulose paper. Low molecular-mass protein standards (Bio-Rad) were used in PAGE and immunoblotting. Rabbit antisera to the protein standards were used to develop the standards (*Carlone* (1986) Anal. Biochem. 155:89-91). Molecular masses were calculated by the method of *Davidson et al.* ((1974) Methods Enzymol. 32:92-102) using appropriate molecular mass standards.

Immunofluorescence Assays. A bacterial suspension containing approximately 400-500 CFU per field (10 µl) was allowed to dry at room temperature on each well of acetone-resistant, 12-well (5 mm diameter), glass slides (25 x 75 mm) (Cel-Line Associates, Newfield, N.J.). The slides were then immersed in acetone for 10 min and air dried at room temperature. MAbs were added to the slides, which were incubated for 30 min at 37°C. After incubation, the slides were gently rinsed with PBS and soaked twice at 5-min intervals, blotted on filter paper, and air dried at room temperature. Fluorescein-labeled rabbit anti-mouse immunoglobulin (courtesy of W. F. Bibb, CDC) was then added, and the slides were incubated for 30 min at 37°C. They were then washed twice with PBS and gently blotted on filter paper. Slides were covered with carbonate-buffered mounting fluid, pH 9.0, and cover slips and were then read with a Leitz Dialux 20 fluorescence microscope equipped with a HBO-100 mercury incident light source, an I cube filter system, a 40x dry objective lens, and 6.3x binoculars (Leitz, Inc., Rockleigh, N.J.)

Immunoelectron-microscopy. Pneumococcal cells were washed two times

with PBS and fixed in a freshly made mixture of 1% paraformaldehyde-0.1% glutaraldehyde for 20 min at 4°C. The cells were dehydrated in a graded alcohol series and then in a 1:1 mixture of absolute ethanol and Lowicryl K4M (Ladd Research Industries, Inc., Burlington, Vt.) for 1 h at 4°C. The cells were pelleted and suspended in a 1:2 mixture of absolute ethanol and Lowicryl K4M for 1 h at 4°C. They were again pelleted and suspended in Lowicryl K4M (undiluted) for 16 h at 4°C. The cells were transferred to fresh and undiluted Lowicryl K4M two times during the next 24-hour period. The Lowicryl K4M-treated cells were imbedded in gelatin capsules and placed in a box lined with aluminum foil. The capsules were hardened using a short-wave UV light source (35 cm distance for 72 h at -20°C). The box was brought to room temperature, and the capsules were allowed to continue hardening for up to 14 days. Samples of the capsule were cut into 100-µm thin sections and picked up on nickel grids. Grids containing the sample were placed on a droplet of ovalbumin solution in PBS containing sodium azide (E. Y. Laboratories, Inc., San Mateo, Calif) for 5 min. The grids (wet) were transferred to a solution of primary MAbs diluted in a solution of BSA reagent (1% BSA in PBS containing 0.1% Triton X- 100, Tween 20, and sodium azide) (E. Y. Laboratories) and incubated for 1 h at room temperature or 18 to 48 h at 4°C in a moist chamber. For antibody binding controls, other grids were wetted with MAbs against *Legionella pneumophila*. The grids were rinsed two times with PBS and incubated on droplets of goat anti-mouse IgG-labeled colloidal gold particles (20 µm)(E. Y. Laboratories) for 1 h at room temperature. The grids were rinsed two times and post-stained with osmium tetroxide, uranyl acetate, and lead citrate. The grids were examined with a Philips 410 transmission electron microscope.

CBA/CaHN/J Mice. X-linked immune deficiency (xid) CBA/N mice as described by Wicker et al., Curr. Top. Microbiol. Immunol. 124:86-101 were used to study the protection afforded by the 37-kDa protein.

#### Example 1. Monoclonal Antibodies

MAbs were produced by the method of Kohler et al. (1975, "Continuous cultures of fused cells secreting antibody of predefined specificity," Nature 256: 495-497) as modified by the method of Zola et al. (1982, "Techniques for production and characterization of monoclonal hybridoma antibodies," in J.G. Hurrell (ed.), Monoclonal hybridoma antibodies: techniques and applications, CRC

Press Inc., Boca Raton, FL, pp. 1-57.) The 37-kDa purified PsaA used for immunization of mice was from *S. pneumoniae* serotype 22F, and had been purified according the method of Tharpe et al. (1996, "Purification and seroreactivity of pneumococcal surface adhesin A (PsaA)," Clin. Diagn. Lab. Immunol. 3: 227-229). All the MAbs were produced by immunizing with purified PsaA from serotype 22F except for 1E7 (1E7A3D7C2), which was produced by immunizing with a nonencapsulated strain of *S. pneumoniae*, R36A (Russell et al., 1990, "Monoclonal antibody recognizing a species-specific protein from *Streptococcus pneumoniae*," J. Clin. Microbiol. 28: 2191-2195). The PsaA was isolated using procedures set forth in Examples 3 and 5 below. BALB/c mice were initially immunized intraperitoneally with purified protein at a final concentration of 180 µg/ml in a 1:1 emulsion with Freund's incomplete adjuvant (Sigma Chemical Co., St. Louis, MO) and phosphate buffered saline pH 7.2. One month later, the mice were boosted with 110 µg/ml purified PsaA without adjuvant. The hybridoma fusion was performed using standard procedures (Clafin et al., 1978, "Mouse myeloma-spleen cell hybrids: enhanced hybridization frequencies and rapid screening procedures," Curr. Top. Microbiol. Immunol. 81: 107-109). Spleen cells from two mice were fused with Sp 2/0-Ag14 myeloma cells (Schulman et al., 1978, "A better cell line for making hybridomas secreting specific antibodies," Nature 276: 269-270). Sera from immunized mice and tissue culture supernatant from hybridized cells were screened for reactivity against PsaA by ELISA using a goat anti-mouse immunoglobulin-horseradish peroxidase conjugate, and by SDS-PAGE combined with Western blotting to standard PsaA, in conventional procedures. Hybridomas yielding positive results in the screen were expanded and used in the identification of the peptides: these were 6F6 (6F6F9C8), 4E9 (4E9G9D3), 8G12 (8G12G11B10), and 1B6 (1B6E12H9). These MAbs, along with 1E7, were used in this investigation.

By means of dot immunoblot and Western blot assays, these MAbs reacted with clinical isolates of *S. pneumoniae* representing the 23 type-specific serotypes present in the licensed pneumococcal polysaccharide vaccine. The Western blots confirmed that the antigen detected had a molecular mass of 37-kDa. In an extended study of 90 serotypes of *S. pneumoniae*, the five MAbs listed in the previous paragraph (but not including 1E7) reacted with 89 of the 90 serotypes (only 1B6 failed to react with serotype 16F). These listed MAbs failed to react

with *E. coli*, respiratory pathogens, or nonpathogens representing 22 genera and 29 species. MAb 1E7 correspondingly reacted with all pneumococcal strains tested (24 serotypes) to yield a sensitivity of 100%. For specificity, none of 55 different nonpneumococcal strains of bacteria (representing 19 genera and 36 species) reacted, thus yielding a specificity of 100%.

When required for use in the experiments described in Example 11, the MAbs were biotinylated by incubating 1 mg of the protein in 0.1 M NaHCO<sub>3</sub>, pH 8.4, with 100 µg of N-hydroxysuccinimidyl-biotin ester (initially dissolved in DMSO).

#### Example 2. Cloning of the Pneumococcal Surface Adhesin A Gene.

*Streptococcus pneumoniae* DNA digested with restriction enzyme *Sau*3A1 was ligated to BamHI digested pUC13 and transformed into *E. coli* TB1. Recombinant clones were identified by colony immunoblot using the 37-kDa monoclonal antibody. The plasmid pSTR3-1 is an example of the pneumococcal surface adhesin A gene cloned into pUC13

#### Example 3. Preparation of Purified 37-kDa Protein Antigen.

Two methods for preparing the 37-kDa protein are to be used. (1) *Streptococcus pneumoniae* is to be conventionally cultured and the cells harvested. Purified 37-kDa protein antigen (pneumococcal surface adhesin A) is to be isolated from the *Streptococcus pneumoniae* cell mass by extraction with a non-ionic detergent and further purified by ammonium sulfate fractionation and isoelectric focusing. (Tharpe et al., 1996. "Purification and seroreactivity of pneumococcal surface adhesin A (PsaA)," Clin. Diagn. Lab. Immunol. 3: 227-229). (2) *E. coli* TB1 strains containing plasmid pSTR3-1 is to be cultured conventionally and the cells harvested. For improved yields, *E. coli* strains, transformed with an expression vector that carries a strong, regulated prokaryotic promoter and which contains the gene coding for the 37-kDa protein, is to be used. Suitable expression vectors are those that contain a bacteriophage λPL Promoter (e.g., pKK1773-3), a hybrid trp-lac promoter (e.g., pET-3a) or a bacteriophage T7 promoter. The 37-kDa protein (PsaA) is then to be extracted from the separated cell mass.

## PROTECTION EXPERIMENTS WITH 37-kDa PROTEIN

Example 4

Twenty CBA/CaHN/J mice carrying the *xid* mutation (x-linked immunodeficiency) were used in this protection study. They were tested for protection against challenge with a virulent capsular type 3 *Streptococcus pneumoniae* strain, WU2. Mice were anesthetized with Ketamine/Rompun and bled infraorbitally to obtain pre-immunization sera. 37-kDa protein (pneumococcal surface adhesin A) was emulsified in complete Freund's adjuvant (CFA) to a protein concentration of 54 µg per ml. Ten mice were injected subcutaneously into 2 axillary and 2 inguinal sites at 0.1 ml per site, delivering approximately 22 µg protein/mouse. Ten control mice were treated identically with CFA and buffer substituting for protein. Fourteen days later, the ten test mice were injected intraperitoneally (IP) with 100 µg of the 37-kDa protein; controls were injected IP with buffer. Eight days following the IP immunizations, all mice were bled infraorbitally to obtain post-immunization sera, and challenged intravenously (IV) with 60 CFU of a log phase culture of *S. pneumoniae* strain WU2. Mice were observed for 21 days, and deaths were recorded. Sera were collected prior to immunizations to establish baseline exposures, and also following the full immunization protocol (but before challenge) in order to correlate circulating antibody to the 37-kDa protein with protection. The results obtained were as follows:

Days post challenge:

- 1: no deaths
- 2: three control mice dead
- 3: two control mice dead
- 4: two control mice dead, one control mouse sick
- 5: one control mouse dead
- 6-21: no mouse deaths

Immunized with 37-kDa protein: 10/10 survived

Controls with no protein: 2/10 survived (8/10 died)

Difference statistically significant: ( $p = 0.0008$ ) Rank sum test.

#### Example 5

Twenty CBA/CaHN/J mice carrying the xid mutation were injected according to the following protocol:

1. All mice were bled prior to immunization to establish baseline immunity. Ten test mice were immunized subcutaneously in four sites with a total of 21  $\mu\text{g}$  of 37-kDa protein antigen (pneumococcal fimbrial protein A) emulsified in Complete Freund's adjuvant (CFA). Ten control mice were immunized identically with CFA and buffer substituting for the antigen.
2. Fourteen days later, the mice were boosted intraperitoneally (IP) with 100  $\mu\text{g}$  of the 37-kDa protein antigen (test mice) or with buffer (controls). No adjuvant was used with this booster immunization.
3. Eight days later, all mice were bled via the infraorbital sinus and the were collected and pooled into the two groups (immunized and controls). At the same time, blood was collected from individual mice to assay for antibody responses.
4. One day later, two additional mice were injected intraocularly with 0.1 ml of pooled immune sera to attempt to passively transfer immunity. Three additional mice were injected intraperitoneally (IP) with 0.1 ml of pooled control mouse sera. (Only five mice were injected at this step because of the small amount of sera obtained from the immunized mice.)



5. One hour after the IP injections, these five mice were challenged intravenously (I.V.) with 140 colony-forming units (CFU) of a mid-log phase *S. pneumoniae* type 3 strain, WU2.

6. At the same time, the eighteen (8 test and 10 control) mice were challenged I.V. with the same culture of WU2.

7. Deaths were tallied daily.

#### RESULTS:

#### No. Dead/Total No. Challenged

Immunized with the 37-kDa protein: 0/8

Control mice: 10/10

#### Passive Protection:

Mice receiving immune sera: 0/2

Mice receiving control sera: 3/3

Mice immunized with the 37-kDa protein were protected from fatal challenge with strain WU2; this immunity could be passively transferred with sera from immunized mice. (Originally 10 test mice were used. However, two of these mice died of other causes prior to being challenged with WU2.)

#### Example 6

An enzyme-linked immunosorbent assay (ELISA) was developed using purified *S. pneumoniae* 37-kDa protein antigen as a capture for human antibodies. Paired sera were tested from children, less than 24 months of age, known to have pneumococcal pneumonia. Disease confirmation was determined by blood culture or antigen in the urine. It was found that 35% (9/26) had antibody titers greater than sera from non-ill children of the same age group,  $p=0.06$ . This illustrates that some of the children responded to the 37-kDa protein antigen after natural infection.

#### Example 7. Preparation of the 37-kDa Protein or Polypeptide Conjugate.

Conjugates can be prepared by use of a carrier protein bound to the 37-kDa protein or polypeptides derived from the 37-kDa protein via a linker, to elicit a T cell dependent response. Such carrier proteins could be any immunogenic protein

such as, for example, keyhole limpet hemocyanin, bovine serum albumin, tetanus toxoid, diphtheria toxoid, or bacterial outer membrane proteins. Examples of bacterial outer membrane proteins, useful as conjugates, include outer membrane proteins of *Neisseria meningitides* and *Haemophilus influenzae*. *Neisseria meningitides* can be selected from *Neisseria meningitides*, group A, B, or C. In addition, the 37-kDa protein or polypeptides thereof can be used in a conjugate where the 37-kDa protein or polypeptides thereof are the T-cell dependent immunogenic carrier for polysaccharide antigens that are B-cell stimulators. This is based on the theory that polysaccharide antigens are B-cell stimulators and that protective immunity is usually generated by a combination of B-cell and T-cell stimulation. Protein antigens exhibit T-cell dependent properties (i.e., booster and carrier priming). T-cell dependent stimulation is important because most children less than two years of age do not respond to T-cell independent antigens. The attachment or conjugation of antigens can be accomplished by conventional processes, such as those described in U.S. Patent No. 4,808,700, involving the addition of chemicals that enable the formation of covalent chemical bonds between the carrier immunogen and the immunogen. In use, the 37-kDa protein antigen of this invention can be administered to mammals, especially humans, in a variety of ways. Exemplary methods include parenteral (subcutaneous) administration given with a nontoxic adjuvant, such as an alum precipitate or peroral administration given after reduction or ablation of gastric activity; or in a pharmaceutical form that protects the antigen against inactivation by gastric juice (e.g., a protective capsule or microsphere). The dose and dosage regimen will depend mainly upon whether the antigen is being administered for therapeutic or prophylactic purposes, the patient, and the patient's history. The total pharmaceutically effective amount of antigen administered per dose will typically be in the range of about 2 µg to 50 µg per patient. For parenteral administration, the antigen will generally be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic and nontherapeutic. Examples of such vehicles include water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Non aqueous vehicles, such as fixed oils and ethyl oleate, may also be used. Liposomes may be used as vehicles. The vehicle may contain

minor amounts of additives, such as substances which enhance isotonicity and chemical stability (e.g., buffers and preservatives).

Example 8.

**Bacterial strains.** All isolates of *S. pneumoniae* were provided and serotyped by the Streptococcal Reference Laboratory, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention (CDC). The pneumococcal serotype 6B strain used for cloning and sequencing was a CDC reference strain (SP-86). *E. coli* DH5 $\alpha$  (Bethesda Research Laboratories, Gaithersburg, MD) was used as the recipient host for plasmids (pUC19 and its derivatives). *S. pneumoniae* strains were grown on Trypticase soy agar plates with 5% sheep blood cells or, where indicated, in Todd-Hewitt broth containing 0.5% yeast extract. *E. coli* cultures were grown in Luria broth which, when required, was supplemented with 100  $\mu$ g/ml of ampicillin (Sigma Chemical Co., St. Louis, Mo.).

**Cloning and sequencing of the *psaA* gene from *S. pneumoniae*, serotype 6B.** A chromosomal library from *S. pneumoniae* serotype 6B was prepared as previously described. (Sampson *et al.* 1994, "Cloning and nucleotide sequence analysis of *psaA*, the *Streptococcus pneumoniae* gene encoding a 37-kilodalton protein homologous to previously reported *Streptococcus* sp. adhesins." Infect. Immun. 62:319-324), except that pUC18 was used as the cloning vector instead of pUC 13. Recombinants were screened by colony immunoblot using monoclonal antibody 1E7. (Russell *et al.* 1990, "Monoclonal antibody recognizing a species-specific protein from *Streptococcus pneumoniae*," J. Clin. Microbiol. 28:2191-2195). This procedure and plasmid purification from positive clones (Ish-Horowitz *et al.* 1981, "Rapid and efficient cosmid cloning," Nucleic Acids Res. 9:2989-2998) and restriction endonuclease analysis, have all been previously described. (Sampson *et al.* 1990, "Nucleotide sequence of *htpB*, the *Legionella pneumophila* gene encoding the 58-kilodalton (kDa) common antigen, formerly designated the 60-kDa common antigen," Infect. Immun. 58:3154-3157; and Sampson *et al.* 1994). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were done as before (Sampson *et al.* 1990). All other DNA manipulations were done according to methods described in Sambrook *et al.* DNA sequencing was performed using the ABI PRISM Dye Terminator Cycle

Sequencing kit and procedure (Perkin-Elmer, Cetus, Foster City, Calif). Sequence data were analyzed with the DNASTAR software program (DNASTAR Inc., Madison, Wis.) and the Wisconsin Genetics Computer Group sequence analysis software program (Fenno *et al.* 1989, "Nucleotide sequence analysis of a type 1 fimbrial gene of *Streptococcus sanguis* FW213," Infect. Immun. 57:3527-3533)

**Preparation of genomic DNA for PCR-RFLP analysis.** High molecular weight pneumococcal DNA was prepared by the procedure of Graves *et al.*, 1993, "Universal bacterial DNA isolation procedure," p.617-621, *in* D.H. Pershing *et al.* (ed), Diagnostic molecular biology. American Society for Microbiology, Washington, D.C.) with modifications. Sixteen-hour cultures of type specific *S. pneumoniae* were grown in 50 ml of Todd-Hewitt broth containing 0.5% yeast extract in screw cap flasks at 37°C without shaking. Cultures were pelleted at 8000 x g for 15 min at room temperature and washed with phosphate-buffered saline (10 mM, pH 7.2). The cell pellet was solubilized in 2.5 ml of buffer composed of 10 mM Tris, 1.0 mM EDTA, pH 8.0, and 0.4% SDS. Fifteen microliters of proteinase K (20 mg/ml) was added, and the lysate was incubated at 37°C for 1 h. The mixture was adjusted to 0.48 M NaCl with the addition of 500 µl of 5M NaCl and, after mixing by inversion, 400 µl of 10% hexadecyltrimethylammonium bromide in 0.7% NaCl was added. This suspension was mixed as before, incubated for 30 min at 65°C, and extracted with an equal volume of phenol-chloroform-isoamyl alcohol. The upper aqueous phase was separated by centrifugation at 1500 x g and extracted with chloroform-isoamyl alcohol. DNA was precipitated from the upper aqueous phase with 2.5 volumes of ethanol at -70°C for 30 min. It was pelleted and dried in a desiccator, resuspended in water and quantitated by measuring absorbance at 260 nm.

**PCR-RFLP.** Restriction enzymes *EcoRI*, *HinfI*, *MaeIII*, *MboII*, *MnII*, and *NheI* were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Id.); *RsaI*, *Tsp509I*, *Eco57I*, and *XmnI* were purchased from New England Biolabs (Beverly, Mass.). Primer sequences for the amplification reaction were selected from the N-terminal (nucleotides 181-201) and C-terminal (nucleotides 1106-1126) sequences of the *S. pneumoniae* serotype 6B gene (P1, AGGATCTAATGAAAAAATTAG (SEQ ID NO:3); P2, TCAGAGGCTTATTTTGCCAAT (SEQ ID NO:4)) and flanking regions. The primers were synthesized using standard procedures.

(i) **DNA amplification** The reactions were performed with the Perkin-Elmer PCR amplification kit. Reaction volumes were 100  $\mu$ l and contained the standard 1x reaction buffer without Mg, 1  $\mu$ M of each primer, 2.0 mM  $MgCl_2$ , 0.2 mM dNTPs, template DNA, and 2.5 U of Taq DNA polymerase. The source of the template DNA was either extracted purified chromosomal DNA or a bacterial colony. Conditions for amplification were as follows: 30 cycles of denaturation 94°C, 1 min., annealing 52°C, 0.5 min., and extension 72°C, 1.5 min. Amplified products were separated on a 1% agarose gel and visualized with ethidium bromide. A direct colony amplification procedure was adapted, which shortened template preparation by eliminating the necessity of extracting chromosomal DNA. The procedure consisted of adding a single bacterial colony directly from the plate into the PCR reaction mixture and heating at 95°C for 10 minutes. The remaining PCR steps were performed as outlined for extracted chromosomal DNA and are given above.

(ii) **Enzyme digestion.** Digestion of amplified products was performed as directed by the manufacturer for the designated enzymes in volumes of 20  $\mu$ l. Digestion products were analyzed by agarose (2% Metaphor agarose, FMC Corp., Rockland, Me.) gel electrophoresis and visualized after being stained with ethidium bromide.

**Analysis of type 6B PsaA.** Genomic DNA was partially digested by *Sau*3A1 was ligated to *Bam*HI-digested pUC18 and used to transform *E. coli* DH5 $\alpha$ . Recombinant colonies were selected for resistance to ampicillin and the formation of white colonies in the presence of isopropyl- $\beta$ -D-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside. Colony immunoblot screening (using anti-PsaA MAb) of approximately 2,500 colonies yielded two positive clones, which were selected, purified, and rescreened by Western blot analysis using the same MAb. They both expressed a protein reactive with MAb to PsaA and which migrated in SDS-PAGE with the expected molecular mass of approximately 37-kDa. One was selected for continued study and was designated pSTR6. Limited restriction enzyme analysis of DNA from the recombinant plasmid showed that the positive clone contained an insert that was 3.5 kb with sites for enzymes *Cl*aI, *E*coRI, and *H*indIII. To localize the PsaA coding region, the insert was double digested with *S*stI (multiple cloning site in vector) and *H*indIII. The resultant fragments were ligated into pUC18 and

transformed into *E. coli* DH5 $\alpha$ . This generated a recombinant containing an insert of about 1.3 kb in size. The resultant subclone pSTR6y, when analyzed by SDS-PAGE and Western blot using anti-PsaA MAb, was shown to express full length PsaA immuno-reactive protein. The complete nucleotide sequence on both strands of the 1.3-kb insert was determined by cycle sequencing of the plasmid subclone using oligonucleotide primers complementary to the sequence. These were made as sequence information became available. The nucleotide sequence of the entire streptococcal insert is set forth in the Sequence Listing as SEQ ID NO: 1. The single open reading frame (ORF) present, beginning at nucleotide 189 and ending at nucleotide 1117, encodes the *psaA* gene sequence. This ORF is 930 nucleotide long and when amplified and subcloned into vector systems such as pGEM (Promega, Madison, Wis.) and BAC-to-BAC<sup>TM</sup> expression system (Bethesda Research Laboratories, Gaithersburg, Md.) expresses full-length PsaA, reactive with anti-PsaA MAb antibodies. This ORF encodes a peptide of 309 amino acids with a deduced molecular weight of 34,598 and an isoelectric point of 5.23. Analysis of the peptide using the algorithm of Kyte *et al.*, 1982, ("A simple method for display of the hydropathic character of a protein." *J. Mol. Biol.* 157:105-132) shows that the peptide contains a major hydrophobic region of 20 amino acids which encodes a putative leader sequence. This leader contains the consensus sequence for signal peptidase cleavage (LXXC). Removal of this leader would result in a peptide of molecular mass 32,465 with a predicted isoelectric point of 4.97. A consensus sequence for a ribosomal binding site (Shine *et al.*, 1974, "The 3'-terminal sequence of *E. coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosomal binding sites." *Proc. Natl. Acad. Sc. USA* 71:1324-1346) is located 5 nucleotides upstream of the ATG start codon.

#### **Comparison of the serotype 6B sequence with streptococcal homologs.**

Comparison of the serotype 6B *PsaA* nucleotide sequence (Bilofsky *et al.* 1988. A GenBank genetic sequence database. *Nucleic Acids Res.* 16:1861-1864) (GenBank accession number U53509) and its flanking regions with the previously published strain R36A *psaA* sequence (Sampson *et al.* 1994. "Cloning and nucleotide sequence analysis of *psaA*, the *Streptococcus pneumoniae* gene encoding a 37-kilodalton protein homologous to previously reported *Streptococcus* sp. adhesins." *Infect. Immun.* 62:319-324) shows the differences between the nucleotide sequences. The computed homology between the two sequences is 74%. Major

areas of discord are in regions upstream and downstream of the ORF and in the initial 60 nucleotide which encode the putative signal peptide. When the two PsaA coding sequences are compared, the sequence homology increases to 78%.

Serotype 6B sequence was also compared to the *psaA* DNA sequence for another vaccine serotype, serotype 2, which was recently submitted to GenBank (Accession number U40786). Computer analysis of these two sequences shows that they are very similar, with computed DNA homology percentages of 99% between the two *psaA* DNA sequences. There are eight single base differences between the two sequences. A comparison of serotype 2 and 6B PsaAs shows almost complete identity; the computed similarity value is 99.3. The eight base difference at the nucleotide level translated into a difference at the peptide level of six amino acids with two of the changes resulting in conservative substitutions. Further analyses and comparisons of the serotype 6B sequence to the other five GenBank PsaA homologues from viridans Streptococci and *E. faecalis* (Fenno *et al.* 1989 "Nucleotide sequence analysis of a type I fimbrial gene of Streptococcus sanguis FW213." Infect. Immun. 57:3527-3533; Sampson *et al.* 1994. "Cloning and nucleotide sequence analysis of *psaA*, the *Streptococcus pneumoniae* gene encoding a 37-kilodalton protein homologous to previously reported Streptococcus sp. adhesins." Infect. Immun. 62:319-324; Ganeshkumar *et al.* 1991. "Nucleotide sequence of a gene coding for a saliva-binding protein (SsaB) from *Streptococcus sanguis* 12 and possible role of the protein in coaggregation with actinomyces" Infect. Immun. 59:1093-1099; Kolenbrander *et al.* 1994. "Nucleotide sequence of the *Streptococcus gordonii* PK488 coaggregation adhesin gene *scaA* and ATP-binding cassette." Infect. Immun. 62:4469-4480; and Lowe *et al.* 1995. "Cloning of an *Enterococcus faecalis* endocarditis antigen: homology with some adhesins from oral streptococci." Infect. Immun. 63:703-706) revealed significant sequence similarity between them. Sequence identities were 81%, 81%, 77%, 82%, and 57%, respectively, for PsaA (*S. pneumoniae* strain R36A), SsaB (*S. sanguis*), FimA (*S. parasanguis*), ScaA (*S. gordonii*) and EfaA (*E. faecalis*). Additionally, all six sequences showed great similarity in organization. They have a hydrophobic leader peptide containing the prolipoprotein consensus sequence LXXC (for signal peptidase II cleavage) within the first 17-20 amino acids. This N-terminal leader sequence appears to represent the area of greatest variability. It is followed by a

region of high similarity from amino acids 36 to 150. The region from 150 to 198 is a variable region and is followed by another conserved region (198 to 309).

**PCR-RFLP analysis of chromosomal DNA from the 23 serotype strains in a 23-valent vaccine.** PCR-RFLP was used to examine the degree of conservation of the gene among 23 *S. pneumoniae* serotypes, representing the 23 serotypes in a 23-valent vaccine. Since previous attempts to amplify pneumococcal type strains with primers corresponding to strain R36A were unsuccessful, primers for PCR were selected from N-terminal and C-terminal sequences of serotype 6B. Using primers complementary to serotype 6B, the *psaA* gene from all 23 serotypes and subtypes represented in the 23-valent vaccine was amplified from chromosomal DNA. A total of 10 enzymes were chosen that had restriction endonuclease digestion sites throughout the entire length of the serotype 6B *psaA* gene. Nine of the 10 enzymes give identical patterns for all 23 *psaA* genes analyzed.

The one exception, restriction enzyme *Tsp509I*, had six sites within the gene and generated seven fragments upon digestion with sizes of 7, 30, 68, 146, 151, 166, and 362 bp. When these fragments are separated on 2% metaphor agarose gel, a five-band pattern can be seen (7- and 30-bp fragments are not seen on these gels because of their small size). For 21 of 23 serotypes this five-fragment enzyme pattern was obtained; but for strains of serotype 4 and 33F, the 146-bp fragment is absent and two new fragments appear flanking the 68-bp fragment making a total of seven bands. This increase in fragment number results from the presence of an extra *Tsp509I* site within the 146-bp fragment. To ascertain the prevalence of this extra site, the *Tsp509I* patterns of 3 to 4 additional strains of each of 23 serotype strains (additional strains of serotype 2 and serotype 25 were not available) were analyzed. All strains analyzed were random clinical isolates from the United States that had been submitted to CDC for serotyping. The majority of the 80 strains were blood isolates; exceptions were 2 from cerebrospinal fluid, 2 from pleural fluid, and 1 each from the eye and nose. Of the strains analyzed, 10% had the extra *Tsp509I* site, resulting in the altered RFLP pattern. This modification was seen only in types 4, 8, 11F, and 33F. In an attempt to determine the prevalence of this altered pattern, the *psaA* gene from 8 additional strains of these 4 types was analyzed for the *Tsp509I* variation (bringing the total to 11-12 for these 4 types). Table 1 summarizes the analyses of serotypes 4, 8, 11A, and 33F. The modified pattern is sporadically present in serotypes 4 and 8, but is essentially always



present in 11 of 12 strains of 11A and all strains of 33F. The occurrence of this pattern could not be correlated with geographic location or region of the United States since strains that showed variation came from diverse regions of the country. All strains of types 4, 8, 11A, and 33F were blood isolates except one 33F strain, which was a nasal isolate; thus the relevance of the site of isolation on prevalence of this modification could not be assessed.

**TABLE 1. Screening of selected serotypes for additional *Tsp5091* restriction site**

Serotype	Ratio of serotypes with additional site to total no. of serotypes tested		Total serotypes with unique patterns
	Expt. #1 <sup>a</sup>	Expt. #2 <sup>b</sup>	% Unique pattern
4	1/3	3/9	33 (4/12) <sup>c</sup>
8	3/4	4/9	44 (7/13)
11A	2/3	5/9	92(11/12)
33F	3/3	( )	100(12/12)

<sup>a</sup> Initial *Tsp5091* analysis which included survey of 2-3 strains each of all 23 vaccine types.

<sup>b</sup> *Tsp5091* analysis of more strains of types showing additional *Tsp5091* site.

<sup>c</sup> Shown in parenthesis is ratio of number with additional site to number tested.

This analysis discloses the cloning and sequencing of the gene encoding PsaA from *S. pneumoniae* serotype 6B and a subsequent analysis of the gene in the 23 pneumococcal polysaccharide vaccine serotypes. Sequence analysis revealed that the serotype 6B sequence and the previously published strain R36A were less similar than expected. The nucleotide sequence and its flanking regions were only 73% homologous to the original strain R36A *psaA*, with the actual PsaA coding sequences had a computed homology of 78%. Protein sequence similarity between the two sequences was only 81%. A comparison of the serotype 6B sequence with the newly submitted serotype 2 pneumococcal *psaA* (a vaccine serotype) gave computed DNA homology values of 99% and 98% protein sequence similarity. These values are evidence of the high sequence conservation for the gene within the vaccine serotypes. Moreover, when the deduced amino acid sequences of these

two sequences were compared with other published sequences for PsaA homologues within the genus, large areas of similarity were evident for all five proteins. Similarity values within the group ranged from 57% to 82%.

The need for a *Streptococcus pneumoniae* vaccine candidate prompted us to clone and sequence the *psaA* gene from *S. pneumoniae* serotype 6B. The heterogeneity between the two pneumococcal *psaA* genes (6B and R36A) led us to examine the vaccine serotypes to determine the degree of diversity among strains. Primers homologous with the N terminus and C terminus of the serotype 6B sequence amplified all 23 of the vaccine serotypes PCR-RFLP analysis using 10 different restriction enzymes representing 21 sites within the serotype 6B gene and shows only one area of diversity, which resulted in an additional *Tsp509I* site for a small number of strains. This study demonstrates that the serotype 6B gene sequence is representative of the sequence found among the vaccine serotypes. Evidence for this includes the 99% DNA sequence identity between serotype 2 and serotype 6B and the uniform and identical restriction patterns covering the 21 sites examined in this study. It is clear that the earlier strain R36A *psaA* sequence represents a variant sequence seemingly not present in the serotypes that were analyzed here since we were unable to amplify them using primers to strain R36A *psaA*. The more important aspect of this study, however, is that there is limited diversity among the vaccine serotypes analyzed. These are the serotypes that cause disease and thus, the ones against which prophylactic measures are needed. The lack of genetic diversity of *psaA* among these serotypes suggests that gene is highly conserved and is an excellent candidate for vaccine development.

#### **Example 9. Monoclonal Antibodies**

The 37-kDa protein from serotype 22F was used to generate monoclonal antibodies 1B6E12H9, 3C4D5C7, 4E9G9D3, 4H5C10F3, 6F6F9C8, and 8G12G11B10 (disclosed in United States Patent Application Ser. No. 08/715,131, incorporated herein by reference). The MAb's were analyzed for their ability to confer protection from infection by *Streptococcus pneumoniae*. Table 2 shows that of 5 monoclonal antibodies tested, one in particular gave efficient protection from subsequent *S. pneumoniae* challenge (8G12G11B10). The protection from *S. pneumoniae* was dose-responsive, demonstrating that the monoclonal antibody was responsible for the protection (Table 3).

**TABLE 2. Passive protection of five Anti-37-kDa monoclonal antibodies in an infant mouse model to *Streptococcus pneumoniae* serotype 6B.**

37-kDa MAb Cell Line <sup>a</sup>	Bacteremia @ 48 h (%)	Death	
		@ 48 h (%)	@ 14 d (%)
1E7A3D7C2	100	100	100
8G12G11B10	100	0	20
4E9G9D3	100	80	100
6F6F9C8	100	60	100
1B6E12H9	100	80	100

<sup>a</sup> Challenge dose ( $1.7 \times 10^3$  cfu) or 10x bacteremic dose 100% ( $BD_{100}$ ). Five/mice group given 50 µg total antibody. All MABs are IgG.

**TABLE 3. Effect of a Second Dose on the Passive Protective Potential of the Anti-37-kDa Monoclonal Antibody 8G12G11B10.**

MAB Dose Level (µg)		Bacteremia @48 h		Death	
Pre	Post <sup>a</sup>	%	Avg. cfu/mi	@48 h %	@10 d %
50	--	100	$1.2 \times 10^4$	0	30
50	50	80	$1.0 \times 10^4$	0	50
5	--	100	$4.7 \times 10^4$	70	100
5	5	100	$3.0 \times 10^4$	50	80
--	--	100	$>10^5$	80	100

<sup>a</sup>All infant mice were challenged with 10 X  $BC_{100}$  ( $2 \times 10^3$  cfu). MAb given 24 h prior to and 24 h after (post-) challenge. 10 mice/group.

### **Example 10. Phage Display Library**

A phage display library containing inserts of 15 amino acid residues located at the N-terminal part of the pIII coat protein (*Parmley and Smith, 1988*) was constructed in the phage FUSE 5 as vector. The library was made by ligating a synthetic 33 bp BglI fragment into FUSE 5 and transfecting *E. coli* Kq1/kan<sup>+</sup> cells by electroporation. The phage progeny contain the display library.

### **Example 11. Screening by Biopanning**

Four cycles of biopanning were carried out for each of the MABs employed in order to screen the phage library of the PsaA peptides prepared in Example 10 (*Smith and Scott, 1993 Meth. Enzymol. 217:228-257*). The substrate of a Petri dish was coated with streptavidin and ten µg of biotinylated anti-PsaA MAB as prepared in Example 1. The remaining biotin binding sites were blocked with 1.5 mL of D-biotin (10 mM). The phage library ( $10^{11}$  to  $10^{12}$  transforming units) was then incubated with the immobilized MAB. Bound phage were eluted from the streptavidin coated plates with 0.1 N HCl, pH 2.2. The eluted phage were titrated and amplified, and then subjected to two further rounds of selection, performed as above. The amount of biotinylated MAB used was 1 nM and 1 pM, respectively, in the second and third rounds, so that only high affinity peptides were bound by the end of the last cycle.

### **Example 12. Amino Acid Sequences of Immunogenic Peptides**

High affinity specimens from the library obtained using the procedures of Example 11 were propagated and sequenced. For each MAB, ten phage specimens resulting from the selection process were sequenced. Approximately 1 µg of single-stranded DNA was purified by phenol and chloroform extraction, ethanol precipitated and resuspended in 7 µL water. Sequencing reactions were performed using a 27-mer primer complementary to the FUSE 5 vector sequence derived from a region in wild-type pIII common to all fd-tet derived vectors and <sup>35</sup>S Sequenase version 2 (U. S. Biochemicals, Cleveland OH). The sequences obtained are shown in Table 4. They were compared to known sequences of PsaA strains 2 and 6B using ClustalV and tFasta programs to identify the epitope on the PsaA with which each peptide is aligned most closely. These epitope positions are also given in Table 4. The peptide obtained using MAB's 8G12, 6F6, and 1E7 align to PsaA

best when an additional residue is present on the protein where the gap appears after residue 13 of the peptide (SEQ ID NO:7 and SEQ ID NO:8).

Table 4. Peptide Sequences Obtained by Biopanning with MAbs

MAb	Sequence	SEQ ID NO:	PsaA Res. Nos.
4E9	TVSRVPWTAWAFHGY	5	132-136
1B6	RSYQHDLRAYGFWRL	6	206-220
8G12	LVRRFVHRRPHVE-SQ	7	252-267
6F6	LVRRFVHHRPHVE-SQ	8	252-267
1E7	LVRRFVHHRPEVE-SQ	8	252-267

**Example 14. Immunization of Mice with Immunogenic Peptides of PsaA**

Peptides having the sequences set out in SEQ ID NOs.: 5, 6, 7, and 8 are to be synthesized in an automated peptide synthesizer. The peptides are to be purified by reversed phase HPLC, and the principal peak is to be collected. Their sequences are to be verified by automated peptide sequencing, using an automated sequencing apparatus such as that manufactured by Beckman Instruments, Inc., Mountain View, CA. Each peptide is to be conjugated to keyhole limpet hemocyanin using coupling mediated by water-soluble carbodiimide reagent. The resulting conjugate is to be dissolved at a final concentration of about 180 µg/ml in phosphate buffered saline pH 7.2 and combined at an approximate 1:1 ratio in emulsion with Freund's incomplete adjuvant (Sigma Chemical Co., St. Louis, MO). BALB/c mice are to be initially immunized intraperitoneally with this suspension, and one month later, the mice are to be boosted with about 110 µg/ml conjugate without adjuvant.

We claim:

1. A peptide that immunospecifically binds to a monoclonal antibody obtained in response to immunizing an animal with *Streptococcus pneumoniae* PsaA.
2. The peptide described in claim 1 wherein the monoclonal antibody is chosen from the group consisting of 1B6E12H9, 3C4D5C7, 4E9G9D3, 4H5C10F3, 6F6F9C8, 8G12G11B10, and 1E7A3D7C2.
3. The peptide described in claim 1 wherein the peptide is 10-25 residues in length.
4. The peptide described in claim 1 wherein the peptide is 12-22 residues in length.
5. The peptide described in claim 1 wherein the peptide is 15 residues in length.
6. The peptide described in claim 1 which is immunogenic against *S. pneumoniae* comprising residues whose sequence is chosen from the group consisting of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, a fragment of SEQ ID NO:5, a fragment of SEQ ID NO:6, a fragment of SEQ ID NO:7, and a fragment of SEQ ID NO:8.
7. A peptide whose sequence results from the method comprising the steps of:
  - (a) providing a library comprised of random oligonucleotides, wherein the oligonucleotides are about 30-45 nucleotides in length;
  - (b) splicing the oligonucleotides of the library into the gene for the gene III coat protein of a filamentous bacteriophage in frame with the codons for the amino acid residues of the coat protein, wherein the gene for the gene III coat protein is contained within the bacteriophage genome, thereby creating a bacteriophage library, and wherein the oligonucleotides are positioned within the gene such that,

when the coat protein is expressed and incorporated into a complete bacteriophage particle, the peptide is available as an epitope to which an antibody can bind:

(c) expanding the bacteriophage library harboring the oligonucleotide library by culturing the bacteriophage library in a host which the bacteriophage infects;

(d) screening the expanded bacteriophage library for a specific bacteriophage particle that immunospecifically reacts with a monoclonal antibody obtained in response to immunizing an animal with *Streptococcus pneumoniae* pneumococcal surface adhesion A protein (PsaA) ; and

(e) sequencing the gene for the coat protein of the specific bacteriophage particle obtained in step (d) thereby yielding the nucleotide sequence of that member of the oligonucleotide library whose translation product has the sequence of the peptide potentially capable of eliciting protective immunity against *Streptococcus pneumoniae*.

8. A therapeutic composition comprising one or more peptides that immunospecifically bind to a monoclonal antibody obtained in response to immunizing an animal with *Streptococcus pneumoniae* PsaA, and an immunostimulatory carrier, wherein the therapeutic composition confers protective immunity against *S. pneumoniae* infection when administered to a subject.

9. The therapeutic composition described in claim 8, wherein at least one peptide is 10-25 residues in length.

10. The therapeutic composition described in claim 8 wherein at least one peptide is 12-22 residues in length.

11. The therapeutic composition described in claim 8 wherein at least one peptide is 15 residues in length.

12. A therapeutic composition comprising one or more peptides that immunospecifically bind to a monoclonal antibody obtained in response to immunizing an animal with *Streptococcus pneumoniae* PsaA and that are immunogenic against *S. pneumoniae*, the peptides comprising residues whose

sequences are chosen from the group consisting of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, a fragment of SEQ ID NO:5, a fragment of SEQ ID NO:6, a fragment of SEQ ID NO:7, and a fragment of SEQ ID NO:8; and an immunostimulatory carrier, wherein the therapeutic composition confers protective immunity against *S. pneumoniae* infection when administered to a subject.

13. A method for conferring protective immunity in a subject against *S. pneumoniae* infection, said method comprising the step of administering to the subject a therapeutic composition comprising one or more peptides that immunospecifically bind to a monoclonal antibody obtained in response to immunizing an animal with *Streptococcus pneumoniae* PsA and that are immunogenic against *S. pneumoniae*, the therapeutic composition further comprising an immunostimulatory carrier.

14. The method described in claim 13, wherein the peptides comprise residues whose sequences are chosen from the group consisting of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, a fragment of SEQ ID NO:5, a fragment of SEQ ID NO:6, a fragment of SEQ ID NO:7, and a fragment of SEQ ID NO:8.

15. A peptide comprising a sequence which is at least 80% identical to a peptide whose sequence is chosen from the group consisting of SEQ ID NO:5 or an immunogenic fragment thereof, SEQ ID NO:6 or an immunogenic fragment thereof, SEQ ID NO:7 or an immunogenic fragment thereof, and SEQ ID NO:8 or an immunogenic fragment thereof.

16. A therapeutic composition comprising one or more of the peptides described in claim 15 and an immunostimulatory carrier, wherein the therapeutic composition confers protective immunity against *S. pneumoniae* infection when administered to a subject.

17. A method for conferring protective immunity in a subject against *S. pneumoniae* infection, comprising the step of administering to the subject the therapeutic composition described in claim 16.



18. A therapeutic composition comprising one or more of the peptides described in claim 15 and an adjuvant, wherein the therapeutic composition confers protective immunity against *S. pneumoniae* infection when administered to a subject.

19. A method for conferring protective immunity in a subject against *S. pneumoniae* infection, comprising the step of administering to the subject the therapeutic composition described in claim 18.

20. A therapeutic composition comprising one or more peptides that immunospecifically bind to a monoclonal antibody obtained in response to immunizing an animal with *Streptococcus pneumoniae* PsA and that are immunogenic against *S. pneumoniae*, the peptides comprising residues whose sequences are chosen from the group consisting of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, a fragment of SEQ ID NO:5, a fragment of SEQ ID NO:6, a fragment of SEQ ID NO:7, and a fragment of SEQ ID NO:8; and an adjuvant, wherein the therapeutic composition confers protective immunity against *S. pneumoniae* infection when administered to a subject.

21. A method for conferring protective immunity in a subject against *S. pneumoniae* infection, said method comprising the step of administering to the subject a therapeutic composition comprising one or more peptides that immunospecifically bind to a monoclonal antibody obtained in response to immunizing an animal with *Streptococcus pneumoniae* PsA and that are immunogenic against *S. pneumoniae*, the therapeutic composition further comprising an adjuvant.

22. The method described in claim 19, wherein the peptides comprise residues whose sequences are chosen from the group consisting of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, a fragment of SEQ ID NO:5, a fragment of SEQ ID NO:6, a fragment of SEQ ID NO:7, and a fragment of SEQ ID NO:8.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Carlone, George M  
Ades, Edwin W  
Sampson, Jacquelyn S  
Tharpe, Jean A  
Zeiler, Joan L  
Westerink, Maria Anna J
- (ii) TITLE OF INVENTION: Epitope Peptides Immuncgenic  
Against Streptococcus Pneumoniae
- (iii) NUMBER OF SEQUENCES: 8
- (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: Fitch, Even, Tabin & Flannery  
(B) STREET: 135 South LaSalle Street, Suite 900  
(C) CITY: Chicago  
(D) STATE: IL  
(E) COUNTRY: USA  
(F) ZIP: 60603-4277
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Diskette  
(B) COMPUTER: IBM Compatible  
(C) OPERATING SYSTEM: Windows  
(D) SOFTWARE: FastSEQ for Windows Version 2.0b
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: Kaba, Richard A  
(B) REGISTRATION NUMBER: 30,562  
(C) REFERENCE/DOCKET NUMBER: 6314/61362
- (ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: 312-372-7842  
(B) TELEFAX: 312-372-7848

(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1330 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 189...1115
- (D) OTHER INFORMATION:

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TACTGCTTCA	GTTTTGGGAC	TCTTTATTGG	CTATAGTTTT	AATGTTGCGG	CAGGTTCTAG	60
TATCGTGCTT	ACAGCTGCTA	GTTTCTTTCT	CATTAGCTTC	TTTATCGCTC	CCAAACAACG	120
ATATTTGAAA	CTGAAAAATA	AACATTGTGT	AAAATAAGGG	GCAAAGCCCT	AATAAATTGG	180
AGGATCTA	ATG AAA AAA	TTA GGT ACA	TTA CTC GTT	CTC TTT CTT	TCT GCA	230
	Met Lys Lys	Leu Gly Thr	Leu Leu Val	Leu Phe Leu	Ser Ala	
	1	5		10		
ATC ATT CTT	GTA GCA TGT	GCT AGC GGA	AAA AAA GAT	ACA ACT TCT	GGT	278
Ile Ile Leu	Val Ala Cys	Ala Ser Gly	Lys Lys Asp	Thr Thr Ser	Gly	
15	20		25		30	
CAA AAA CTA	AAA GTT GTT	GCT ACA AAC	TCA ATC ATC	GCT GAT ATT	ACT	326
Gln Lys Leu	Lys Val Val	Ala Thr Asn	Ser Ile Ile	Ala Asp Ile	Thr	
	35		40		45	
AAA AAT ATT	GCT GGT GAC	AAA ATT GAC	CTT CAT AGT	ATC GTT CCG	ATT	374
Lys Asn Ile	Ala Gly Asp	Lys Ile Asp	Leu His Ser	Ile Val Pro	Ile	
	50		55		60	
GGG CAA GAC	CCA CAC GAA	TAC GAA CCA	CTT CCT GAA	GAC GTT AAG	AAA	422
Gly Gln Asp	Pro His Glu	Tyr Glu Pro	Leu Pro Glu	Asp Val Lys	Lys	
	65		70		75	
ACT TCT GAG	GCT GAT TTG	ATT TTC TAT	AAC GGT ATC	AAC CTT GAA	ACA	470
Thr Ser Glu	Ala Asp Leu	Ile Phe Tyr	Asn Gly Ile	Asn Leu Glu	Thr	
	80		85		90	
GGT GGC AAT	GCT TGG TTT	ACA AAA TTG	GTA GAA AAT	GCC AAG AAA	ACT	518
Gly Gly Asn	Ala Trp Phe	Thr Lys Leu	Val Glu Asn	Ala Lys Lys	Thr	
	95		100		105	110

GAA AAC AAA GAC TAC TTC GCA GTC AGC GAC GGC GTT GAT GTT ATC TAC Glu Asn Lys Asp Tyr Phe Ala Val Ser Asp Gly Val Asp Val Ile Tyr 115 120 125	566
CTT GAA GGT CAA AAT GAA AAA GGA AAA GAA GAC CCA CAC GCT TGG CTT Leu Glu Gly Gln Asn Glu Lys Gly Lys Glu Asp Pro His Ala Trp Leu 130 135 140	614
AAC CTT GAA AAC GGT ATT ATT TTT GCT AAA AAT ATC GCC AAA CAA TTG Asn Leu Glu Asn Gly Ile Ile Phe Ala Lys Asn Ile Ala Lys Gln Leu 145 150 155	662
AGC GCC AAA GAC CCT AAC AAT AAA GAA TTC TAT GAA AAA AAT CTC AAA Ser Ala Lys Asp Pro Asn Asn Lys Glu Phe Tyr Glu Lys Asn Leu Lys 160 165 170	710
GAA TAT ACT GAT AAG TTA GAC AAA CTT GAT AAA GAA AGT AAG GAT AAA Glu Tyr Thr Asp Lys Leu Asp Lys Leu Asp Lys Glu Ser Lys Asp Lys 175 180 185 190	758
TTT AAT AAG ATC CCT GCT GAA AAG AAA CTC ATT GTA ACC AGC GAA GGA Phe Asn Lys Ile Pro Ala Glu Lys Lys Leu Ile Val Thr Ser Glu Gly 195 200 205	806
GCA TTC AAA TAC TTC TCT AAA GCC TAT GGT GTC CCA AGT GCC TAC ATC Ala Phe Lys Tyr Phe Ser Lys Ala Tyr Gly Val Pro Ser Ala Tyr Ile 210 215 220	854
TGG GAA ATC AAT ACT GAA GAA GAA GGA ACT CCT GAA CAA ATC AAG ACC Trp Glu Ile Asn Thr Glu Glu Glu Gly Thr Pro Glu Gln Ile Lys Thr 225 230 235	902
TTG GTT GAA AAA CTT CGC CAA ACA AAA GTT CCA TCA CTC TTT GTA GAA Leu Val Glu Lys Leu Arg Gln Thr Lys Val Pro Ser Leu Phe Val Glu 240 245 250	950
TCA AGT GTG GAT GAC CGT CCA ATG AAA ACT GTT TCT CAA GAC ACA AAC Ser Ser Val Asp Asp Arg Pro Met Lys Thr Val Ser Gln Asp Thr Asn 255 260 265 270	998
ATC CCA ATC TAC GCA CAA ATC TTT ACT GAC TCT ATC GCA GAA CAA GGT Ile Pro Ile Tyr Ala Gln Ile Phe Thr Asp Ser Ile Ala Glu Gln Gly 275 280 285	1046
AAA GAA GGC GAC AGC TAC TAC AGC ATG ATG AAA TAC AAC CTT GAC AAG Lys Glu Gly Asp Ser Tyr Tyr Ser Met Met Lys Tyr Asn Leu Asp Lys 290 295 300	1094
ATT GCT GAA GGA TTG GCA AAA TAAGCCTCTG AAAACGTCA TTCTCATGTG AGCT Ile Ala Glu Gly Leu Ala Lys 305	1149

```

GGCGTTTTTT CTATGCCAC ATTTCCGGTC AAATCATTGG AAAATTCTGA CTGTTTCAGA 1209
TACAATGGAA GAAAAAGAT TGGAGTATCC TATGGTAACT TTTCTCGGAA ATCCTGTGAG 1269
CTTTACAGGT AAACAACTAC AAGTCGGCGA CAAGGCGCTT GATTTTCTC TTACTACAAC 1329
A 1330

```

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 309 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Lys Lys Leu Gly Thr Leu Leu Val Leu Phe Leu Ser Ala Ile Ile
 1           5           10           15
Leu Val Ala Cys Ala Ser Gly Lys Lys Asp Thr Thr Ser Gly Gln Lys
 20           25           30
Leu Lys Val Val Ala Thr Asn Ser Ile Ile Ala Asp Ile Thr Lys Asn
 35           40           45
Ile Ala Gly Asp Lys Ile Asp Leu His Ser Ile Val Pro Ile Gly Gln
 50           55           60
Asp Pro His Glu Tyr Glu Pro Leu Pro Glu Asp Val Lys Lys Thr Ser
 65           70           75           80
Glu Ala Asp Leu Ile Phe Tyr Asn Gly Ile Asn Leu Glu Thr Gly Gly
 85           90           95
Asn Ala Trp Phe Thr Lys Leu Val Glu Asn Ala Lys Lys Thr Glu Asn
100           105           110
Lys Asp Tyr Phe Ala Val Ser Asp Gly Val Asp Val Ile Tyr Leu Glu
115           120           125
Gly Gln Asn Glu Lys Gly Lys Glu Asp Pro His Ala Trp Leu Asn Leu
130           135           140
Glu Asn Gly Ile Ile Phe Ala Lys Asn Ile Ala Lys Gln Leu Ser Ala
145           150           155           160
Lys Asp Pro Asn Asn Lys Glu Phe Tyr Glu Lys Asn Leu Lys Glu Tyr
165           170           175
Thr Asp Lys Leu Asp Lys Leu Asp Lys Glu Ser Lys Asp Lys Phe Asn
180           185           190
Lys Ile Pro Ala Glu Lys Lys Leu Ile Val Thr Ser Glu Gly Ala Phe
195           200           205
Lys Tyr Phe Ser Lys Ala Tyr Gly Val Pro Ser Ala Tyr Ile Trp Glu
210           215           220
Ile Asn Thr Glu Glu Glu Gly Thr Pro Glu Gln Ile Lys Thr Leu Val
225           230           235           240
Glu Lys Leu Arg Gln Thr Lys Val Pro Ser Leu Phe Val Glu Ser Ser
245           250           255
Val Asp Asp Arg Pro Met Lys Thr Val Ser Gln Asp Thr Asn Ile Pro

```

260 265 270  
 Ile Tyr Ala Gln Ile Phe Thr Asp Ser Ile Ala Glu Gln Gly Lys Glu  
 275 280 285  
 Gly Asp Ser Tyr Tyr Ser Met Met Lys Tyr Asn Leu Asp Lys Ile Ala  
 290 295 300  
 Glu Gly Leu Ala Lys  
 305

## (2) INFORMATION FOR SEQ ID NO:3:

## (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: Genomic DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGGATCTAAT GAAAAAATTA G

21

## (2) INFORMATION FOR SEQ ID NO:4:

## (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: Genomic DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCAGAGGCTT ATTTTGCCAA T

21

## (2) INFORMATION FOR SEQ ID NO:5:

## (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Thr Val Ser Arg Val Pro Trp Thr Ala Trp Ala Phe His Gly Tyr  
 1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Arg	Ser	Tyr	Gln	His	Asp	Leu	Arg	Ala	Tyr	Gly	Phe	Trp	Arg	Leu
1				5					10					15

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Leu	Val	Arg	Arg	Phe	Val	His	Arg	Arg	Pro	His	Val	Glu	Ser	Gln
1				5					10					15

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Leu	Val	Arg	Arg	Phe	Val	His	His	Arg	Pro	His	Val	Glu	Ser	Gln
1				5					10					15

# INTERNATIONAL SEARCH REPORT

National Application No  
PC1/US 99/04326

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C07K14/315 C07K7/08 A61K38/10 A61K39/09  
G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CROOK, JENNIFER ET AL: "Immunoreactivity of five monoclonal antibodies against the 37-kilodalton common cell wall protein ( PsaA ) of Streptococcus pneumoniae" CLIN. DIAGN. LAB. IMMUNOL. (1998), 5(2), 205-210, 1998, XP002109323 the whole document ---	1-22
Y	THARPE, JEAN A. (1) ET AL: "Purification and seroreactivity of pneumococcal surface adhesin A ( PsaA." CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, (1996) VOL. 3, NO. 2, PP. 227-229., XP002109329 the whole document --- -/--	1-22

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

20 July 1999

Date of mailing of the international search report

03/08/1999

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Authorized officer

Hix, R



# INTERNATIONAL SEARCH REPORT

International Application No  
PCI/US 99/04326

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SAMPSON, JACQUELYN S. (1) ET AL: "Limited diversity of Streptococcus pneumoniae psaA among pneumococcal vaccine serotypes." INFECTION AND IMMUNITY, (1997) VOL. 65, NO. 5, PP. 1967-1971. , XP002109325 the whole document ----	1-22
Y	SAMPSON, JACQUELYN S. (1) ET AL: "Cloning and nucleotide sequence analysis of psaA, the Streptococcus pneumoniae gene encoding a 37-kilodalton protein homologous to previously reported Streptococcus sp. adhesins." INFECTION AND IMMUNITY, (1994) VOL. 62, NO. 1, PP. 319-324. , XP002109326 the whole document ----	1-22
A	WO 93 10238 A (US HEALTH) 27 May 1993 (1993-05-27) cited in the application the whole document ----	
A	WO 91 15237 A (UNIV SASKATCHEWAN) 17 October 1991 (1991-10-17) cited in the application the whole document ----	
A	TALKINGTON, DEBORAH F. (1) ET AL: "Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA)." MICROBIAL PATHOGENESIS, (1996) VOL. 21, NO. 1, PP. 17-22. , XP002109327 cited in the application the whole document ----	
A	H. RUSSELL ET AL.: "Monoclonal antibody recognizing a species-specific protein from Streptococcus pneumoniae." J. CLINICAL MICROBIOLOGY, vol. 28, no. 10, October 1990 (1990-10), pages 2191-2195, XP002109328 cited in the application the whole document -----	

# INTERNATIONAL SEARCH REPORT

national application No.

PCT/US 99/ 04326

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim(s) 13, 14, 17, 19 and 21  
is(are) directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

information on patent family members

Intr. National Application No

PCI/US 99/04326

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